

Faculty of Medicine - Doctoral School in Health Sciences

Doctoral Programme in Clinical Research

A study on genetic mutations involving mitochondrial disorders – a diagnostic approach and application of human iPSCs to understand disease pathogenesis

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ACADEMIC DISSERTATION

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Cover Page Pictures:

Left – Labelling (green) mitochondria of live primary human fibroblasts cells

Right - Electron microscope atlas - Mitochondria of hiPSC- RPE cells

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- *To my loving Parents*

*Nothing in life is to be feared, it is only to be understood. Now is the time to
understand more, so that we may fear less - Marie Curie*

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ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals I, II and III.

- I. *Roomets, E., ***Polinati, P.P.**, Euro, L., Eskelin, P.M., Paganus, A. and Tyni, T., 2012. Carrier frequency of a common mutation of carnitine palmitoyltransferase 1A deficiency and long-term follow-up in Finland. *The Journal of pediatrics*, **160**(3), 473-479.e1.

***Equal Contribution**

- II. Ojala, T., **Polinati, P.**, Manninen, T., Hiippala, A., Rajantie, J., Karikoski, R., Suomalainen, A. and Tyni, T., 2012. New mutation of mitochondrial DNAJC19 causing dilated and noncompaction cardiomyopathy, anemia, ataxia, and male genital anomalies. *Pediatric research*, **72**(4), 432-437.
- III. **Polinati, P.**, Ilmarinen, T., Trokovic, R., Hyotylainen, T., Otonkoski, T., Suomalainen, A., Skottman, H. and Tyni, T.A., 2014. Patient specific induced pluripotent stem cells derived RPE cells: understanding the pathogenesis of retinopathy in LCHAD deficiency. *Invest Ophthalmol Vis Sci*. 2015 May 1;56(5):3371-82 doi: 10.1167/iovs.14-14007.

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ABBREVIATIONS

2D-PAGE	Two - dimensional polyacrylamide gel-electrophoresis
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
ACADM	Medium -chain acyl CoA dehydrogenase
ACADVL	Very long- chain acyl CoA dehydrogenase
ACAD9	Acyl CoA dehydrogenase 9
ARPE19	a Human retinal pigment epithelial cell line
BMP	Bone morphogenic factor
BN-PAGE	Blue native polyacrylamide gel electrophoresis
CACT	Carnitine acylcarnitine translocase
Cer	Ceramide
CL	Cardiolipin
CPT	Carnitine palmitoyl transferase
CRALBP	Cellular retinaldehyde-binding protein
<i>CPT1A</i>	<i>CPT1A</i> gene/variant
DAPI	4',6-diamidino-2-phenylindole
DCMA	Dilated cardiomyopathy with ataxia syndrome
DHA	Docosaehaenoic acid
DNA	Deoxy ribonucleic acid
DNAJC19	DnaJ (Hsp40 homolog) subfamily C, member 19
dNTP	Deoxynucleotide triphosphates
DAPI	4',6-diamidino-2-phenylindole
EBs	Embryoid body
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal-transition
ERG	Electroretinogram
ESC	Embryonic stem cell
FABP	Fatty acid binding protein
FAT	Fatty acyl translocase
FATP	Fatty acid transport proteins

FAO	Fatty acid oxidation
FA β O	Fatty acid β oxidation
FA	Fatty acid
FAD	Flavin adenine dinucleotide
FADH	Reduced form of FAD
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HADHA	Hydroxyacyl-CoA Dehydrogenase, alpha subunit
HELLP	Haemolysis, Elevated Liver enzymes, Low Platelet count
hESC	Human embryonic stem cell
Hh	Hedgehog protein
Hsp	Heat shock protein
hiPSC	Human induced pluripotent stem cell
IMM	Inner mitochondrial membrane
IMS	Inner membrane space
iPSC	Induced pluripotent stem cells
KO	Knock Out
Klf	Kruppel-like factor
LCHAD	Long chain acyl CoA dehydrogenase
LCHADD	Long chain acyl-CoA dehydrogenase deficiency
MAGMAS	Mitochondria-associated protein involved in granulocyte-macrophage colony-stimulating factor signal transduction
mtDNA	Mitochondrial DNA
MEF	Mitomycin-inactivated mouse Embryonic Fibroblasts
MITF	Microphthalmia-associated transcription factor
NADH	Reduced form of NAD
NAD	Nicotinamide adenine dinucleotide
OAT	Ornithine δ aminotransferase
OCT4	Octamer-binding transcription factor 4
OMM	Outer mitochondrial membrane
OTX 2	Orthodenticle homeobox-2
OXPHOS	Oxidative phosphorylation

PAX	Paired box protein-6
PBS	Phosphate buffered saline
PHB	Prohibitin
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PEDF	Pigment epithelium-derived factor
POS	Photoreceptor outer segments
PRE	Premelanosome protein
RC	Respiratory chain
RCS	Royal College of Surgeon
RNA	Ribonucleic acid
RPE	Retinal pigment epithelium
RP	Retinitis pigmentosa
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SRX	Sex determining region Y
TALEN	Transcription activator-like-effect or nucleases
TAZ	Tafazzin protein encoded by <i>TAZ</i> gene
TER	Transepithelial electric resistance
TFP	Mitochondrial trifunctional protein
TG	Triglycerides
TIM	Inner membrane translocase
TOM	Outer membrane translocase
TZ	Tafazzin
VEGF	Vascular endothelial growth factor
VDAC	Voltage-dependent anion channel
ZFN	Zinc finger nucleases
ZO	Zona occludens

ABSTRACT

Mitochondrial diseases are generally caused by genetic variants that may affect cell function during the process of energy generation: right from the start of protein translocation to the fatty acid degradation by beta-oxidation (β -oxidation). The main objective of this PhD thesis is to study genetic variants that cause mitochondrial diseases and also to understand the disease pathogenesis of a known disease using the induced pluripotent stem cell (iPSC) method, a revolutionary approach in regenerative medicine.

In the first study, we carried out a long-term follow up of six metabolic diseased patients and subsequently we performed a carrier frequency study of the identified carnitine palmitoyl transferase 1A (*CPT1A*) gene variant in the Finnish population. We identified a novel homozygous variant c.1364A>C (p.Lys455Thr) in exon 12 of the *CPT1A* gene. No carriers of the variant c.1364A>C were detected upon minisequencing of 150 control samples but the allele frequency of *CPT1A* variant in global population is 0.0002142 (ExAC Browser) whereas in the Finnish population (6614 allele number) the frequency is 0.001966. The identified variant was predicted to cause improper folding of the CPT1A protein, which leads to its degradation. All patients were treated with a high-carbohydrate and a low fat diet.

In the second study, we focused on the human DnaJ (Hsp40 homolog) subfamily C, member 19 (DNAJC19) deficiency. Our studies showed that it causes early onset dilated cardiomyopathy syndrome (DCMA). This is the first report of a genetic defect in the mitochondrial protein, DNAJC19, outside of the Canadian Dariusleut Hutterite population. This defect is characterized by an unusual aetiology for an early onset recessively inherited dilated cardiomyopathy that is associated with ataxia and male genital anomalies. Sequencing of the human *DNAJC19* gene revealed a homozygous single nucleotide (A) deletion in exon 6 that cause a frameshift and lead to the premature termination of the protein.

In the third study, the pathogenesis of retinopathy in long-chain acyl-CoA dehydrogenase deficiency (LCHADD) was studied using iPSC technology. Retinopathy is an unusual manifestation of LCHADD, as mitochondrial fatty acid β -oxidation (FA β O) has not been considered to play a major role in the metabolism of the retina. Among all defects of mitochondrial FA β O, only long-chain acyl-CoA dehydrogenase (LCHAD) and mitochondrial trifunctional protein (TFP) deficiencies have developed pigmentary retinopathy and peripheral neuropathy. We elucidated how a genetic variant in the FA β O cycle can disrupt the retinal pigment epithelium (RPE) that can eventually lead to blindness. In addition, we developed a new *in vitro* cell model; iPSC clones were generated from LCHADD patient fibroblasts and further differentiated into RPE cells. Several changes were observed in patient RPE cells such as decreased cell size, lower pigmentation and irregular pattern of morphology. Electron microscopy analysis showed an accumulation of a few melanosomes, more melanolysosomes, and large sized lipid

droplets in patient RPE cells. Furthermore, increased levels of triglycerides in patient RPE cells were observed upon mass spectrometric analysis. We concluded that all these changes had contributed to the disruption of the RPE layer that leads to blindness in LCHAD deficiency patients.

Finally, the research done for this thesis succeeded in identifying novel variants in *CPT1A* and *DNAJC19* genes in Finnish patients. Our long-term follow up studies on CPT1A deficiency can help patients in better diagnosis, which further helps clinicians to identify the genetic cause. We also found a novel phenotype with DNAJC19 deficiency. Further we established the groundwork to understand the pathogenesis of retinopathy in LCHADD patients using an advanced method that helps to study in depth pathogenesis mechanism.

1 INTRODUCTION

Generally a metabolic disease or disorder is a condition in which the disruption of normal metabolism happens at the cellular level. A plethora of key enzymes have been demonstrated to play key roles in various interdependent metabolic pathways. Most of the metabolic disorders results in cellular dysfunction thereby causing disturbances to critical biochemical reactions. All inherited metabolic diseases include both autosomal recessive and autosomal dominant, the onset of symptoms appear under conditions when the body metabolism undergoes stressful conditions such as prolonged fasting or during illness (Bennett et al. 2004).

The most common group of metabolic disorders comprises various mitochondrial diseases that have an estimated prevalence of 1 in 5000 (Elliott et al. 2008). They may manifest a wide range of symptoms and are not confined to a particular organ or tissue. Mitochondrial diseases may also have onset at any age with any mode of inheritance. In particular they mainly affect energy demanding tissues such as skeletal muscle, heart, and brain and in addition may also affect the peripheral nerves and also the eyes.

Mitochondria are the ‘power houses’ of the cells as they are responsible for generating energy in the form of adenosine triphosphate (ATP). Mitochondrial fatty acid β -oxidation (FA β O) is an important pathway under fasting conditions when the glucose supply is limited. A number of enzymes involved in this pathway mediate the generation of the required amount of energy for tissues especially for the muscle and heart particularly during the non-fed/fasting state. Impairment in the function of any one of these enzymes can have a devastating effect that could lead to a FA β O disorder, in most cases autosomal recessively inherited. The classical clinical manifestation of a FA β O disorder is a metabolic crisis with hypoketotic hypoglycaemia and fat accumulation in tissues particularly in the liver, skeletal muscle and heart, during the first years of life. Without an emergency treatment, these patients may die a sudden death during the neonatal stage or in infancy. For these reasons, it has become the norm to screen FA β O disorders in neonates by mutation analysis or acylcarnitine analysis (Gillis et al. 2002).

Various treatment strategies of β -oxidation defects currently include a therapeutic intervention that secures sufficient caloric intake during periods of metabolic stress and fasting. Another primary treatment method contributes to preventing the accumulation of β -oxidation intermediates by providing only the required amount of essential fatty acids (FA's). Currently, the dietary regime strategy for FA β O deficiencies primarily seeks avoidance of fasting and restriction of dietary fat containing long-chain fatty acids. A low fat diet approach in addition to the inclusion of essential fatty acid and fat-soluble vitamin supplements in the diet is important (Munnich 1992).

The research in this thesis summarizes novel genetic variants that result in mitochondrial metabolic disorders in the Finnish population. It also provides a brief description of a clinical study and genetic analysis of mitochondrial metabolic diseases for clinicians. We present two novel disease-causing variants in carnitine palmitoyl transferase 1A (*CPT1A*) and DnaJ (Hsp40 homolog) subfamily C, member 19 (*DNAJC19*) in the Finnish population and also focus on understanding the pathogenesis of retinopathy condition under long-chain acyl-CoA dehydrogenase deficiency (LCHADD). Both long-chain acyl-CoA dehydrogenase (LCHAD) and mitochondrial trifunctional protein (TFP) deficiencies have unusual manifestations of a β -oxidation defects including: progressive retinopathy and peripheral neuropathy, both of which may present in childhood or adulthood.

We were able to provide genetic counselling and better treatment for patients with FA β O disorders by investigating the genetic cause through detecting new variants. The identification of the underlying mechanisms that cause typical manifestations by using new *in vitro* models will also help to provide new therapies.

2 REVIEW OF THE LITERATURE

2.1 Mitochondria and their evolutionary origin

It is believed that the mitochondria played a vital role in the evolution of eukaryotic cells. Mitochondria are organelles that have their own genome comprising a small circular plasmid DNA, thus strongly supporting the theory of endosymbiosis. In addition, the double membrane structure of the mitochondria closely resembles prokaryotic characteristics such as in bacteria. Mitochondria are believed to have originated by the endosymbiosis of ancient bacteria (Margulis et al. 1998, Cavalier 2006). The endosymbiotic theory hypothesizes that mitochondria evolved from the engulfment of an alpha-proteobacterium (Lane & Martin 2010) by an early eukaryotic cell (Wallace 2005).

Mitochondria act as the cell's power house and generate energy in the form of ATP. The size of the mitochondria ranges from 1-10 μm . The number of mitochondria in cells varies from a single mitochondrion in the retinal cell to thousands of mitochondria in hepatocytes. The shape of the mitochondria also varies depending on the cell type, ranging from tubular networks to punctuated structures such as sheets and spheres (Figure 1) (Perkins et al. 2001, Scheffler 2001).

2.2 The structure of the mitochondria

The mitochondrion is the largest organelle in the cell. It consists of two submitochondrial compartments, namely the outer membrane (OM) and the inner membrane (IM) with its cristae and the matrix, or central compartment. The space between the OM and IM is the inner membrane space (IMS) (Figure 1). Most of the mitochondrial proteins that are needed for the normal functions of the mitochondria are encoded by nuclear genes (Scheffler 2001).

2.2.1 The outer mitochondrial membrane

The outer mitochondrial membrane (OMM) comprises of approximately 50% of phospholipids and 50% of proteins. The OMM contains a protein called the voltage-dependent anion channel (VDAC) or porin. They are capable of permeating molecules having a molecular mass up to 5 kDa (Schleiff & Becker 2011). VDAC has a β -barrel structure similar to bacterial proteins and they belong to the porin family. Ions, nutrient

molecules, adenosine triphosphate (ATP), adenosine diphosphate (ADP), etc. can easily pass through the OMM (Schein et al. 1976; Wurm et al. 2011).

2.2.2 The inner mitochondrial membrane

The inner mitochondrial membrane (IMM) comprised of approximately 20% phospholipid and 80% of protein. The IMM is the site for synthesis and localization of a unique four fatty acyl chain phospholipid, cardiolipin. This makes the IMM impermeable to ions and solutes (Fadeel et al. 2009), a vital function for the constant maintenance of the electrochemical potential that is necessary to mediate the synthesis of ATP in mitochondria. The transporter proteins such as inner membrane translocase (TIM) or adenine nucleotide translocase (ANT) are embedded in the IMM, they are critical for shuttling proteins and metabolites between the matrix and the inter membrane space. As the surface of the IMM is larger than the outer membrane, the inner membrane (Figure 1) forms numerous invaginations or cristae that protrude into the matrix (Zick et al. 2009). The mitochondrial matrix also contains enzymes needed for the citric acid cycle, mtDNA, ribosomes and also functions as a site for calcium storage.

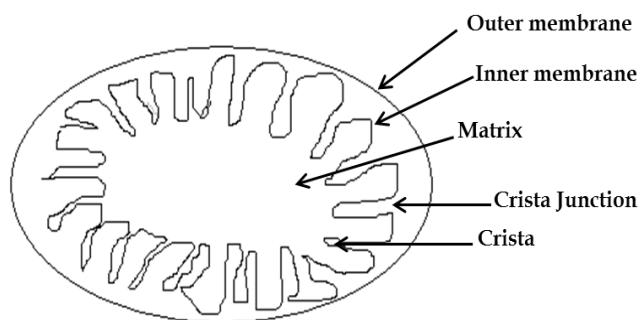


Figure 1 - Schematic representation of mitochondrial structure

2.3 Functions of mitochondria

Mitochondria are extremely critical organelles whose main function is to produce metabolic energy by the oxidation of carbohydrates and fatty acids. The metabolic end product of sugars and fats is acetyl-coenzyme A, which enters into 'Krebs cycle' (also known as the Citric acid cycle or Tricarboxylic acid cycle) where it is further oxidized resulting in the reduction of nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD) coupled with the generation of ATP. Thereafter these are further re-oxidized in the respiratory chain (RC) or oxidative phosphorylation

(OXPHOS) by a series of reactions that are coupled to the synthesis of ATP (Yip et al. 2011).

2.3.1 Lipids

Lipids are the main sources for storage of energy, membranes building, signalling, environmental sensing, and protecting cells from highly reactive chemicals (Harvey 2004). Fatty acids (FA's) are components of phospholipids, sphingolipids, triglycerides and diacyl glycerols all of which are major lipids in the cell. A single FA consists of a long hydrocarbon chain with a carboxyl group at one end. A saturated FA (e.g. palmitate) has only single bonds, between the adjacent carbon atoms, whereas an unsaturated fatty acid (e.g. C20 arachidonate) has one or more double bonds between certain carbon atoms in the hydrocarbon chain and results in various degrees of unsaturation.

2.3.1.1 The mitochondrial β -oxidation of fatty acids

Triglycerides are type of fat found in blood. When body has enough of calories the left over fat is stored in the form of triglycerides. After release of fatty acids from triglycerides, lipoproteins act as transporters by carrying them and further binding them to albumin (Mitchell & Hatch 2011). These fatty acid albumin complexes are transported into the blood stream. Later, the FA's dissociate from albumin, to be taken up by the various tissues in the body (Pohl et al. 2004). The fatty acids that enter into the cells are either esterified for storage or transported directly to the mitochondria for β -oxidation of the FAs. There are two theories on how FAs are transported across cell membranes and imported into cells. The first theory is that, FAs can cross lipid bilayers, independent of proteins by passive diffusion using a 'flip flop' mechanism. The second theory states that the fatty acids enter cells facilitated by a specific protein-mediated transport mechanism, using the fatty acid transport proteins (FATP), fatty acid binding protein (FABP), fatty acid translocase/CD36 (FAT) and Caveolin-1. (Mitchell & Hatch 2011)

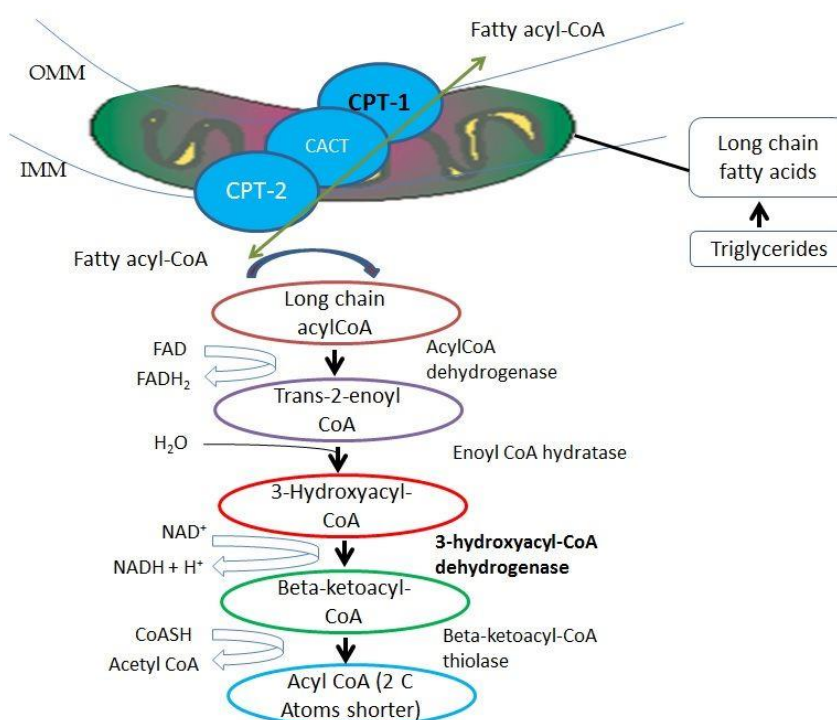


Figure 2 - Schematic illustrations of fatty acid transport and steps in the β -oxidation cycle (Variants examined in this thesis are in Bold letters *CPT1A* and *LCHAD* (3-hydroxyacyl-CoA dehydrogenase))

2.3.1.2 Entry into the β -oxidation of FAs

Short chain fatty acids, FAs with 10 carbons or less, can enter a mitochondrial membrane freely through the outer and inner mitochondrial membranes. The acyl carnitine shuttle is necessary for transmembrane transport of long chain fatty acids into the mitochondrion. The mitochondrial matrix contains enzymes that are needed for fatty acid β -oxidation. Mitochondrial fatty acid β -oxidation (FA β O) can theoretically be divided into two steps: (a) the transfer of acyl groups into the mitochondrion for oxidation and (b) shortening of intramitochondrial chain by oxidative removal of two-carbon (acetyl) units (Figure 2) (Kiens et al. 2011).

The acyl carnitine shuttle is necessary for transportation of long chain fatty acids and it is composed of carnitine and three enzymes: carnitine palmitoyltransferase (CPT) I and II, and carnitine acylcarnitine translocase (CACT) (Figure 2). CPT I facilitates the transfer of acyl groups from CoA to carnitine at the outer mitochondrial membrane, CACT is responsible for the exchange of acylcarnitines to carnitine across the inner mitochondrial membrane and CPT II catalyzes the transfer of acyl groups from carnitine

back to CoA. Carnitine is obtained primarily from dietary sources but is also synthesized by the liver and by the kidney (Kiens et al. 2011).

2.3.1.3 Steps in the mitochondrial FA β -oxidation cycle

When fatty acyl-CoA enters the β -oxidation cycle in the mitochondria it undergoes 3 steps: oxidation, hydration and cleavage. Initially, fatty acyl-CoA is oxidized to a trans alkene i.e. enoyl-CoA by acyl-CoA dehydrogenase with the help of the prosthetic group FAD. Next, the transenoyl-CoA is hydrated to L-3-hydroxyacyl-CoA through the enzyme enoyl CoA hydratase. The alcohol group in hydroxyacyl-CoA is then oxidized by NAD⁺ to a carbonyl group by the hydroxyacyl-CoA dehydrogenase enzyme to form 3-ketoacyl-CoA. NAD⁺ is utilized instead of FAD to oxidize alcohol. Eventually 3-ketoacyl-CoA is converted to acyl-CoA and acetyl-CoA by thiolase enzyme. Acyl-CoA is cleaved off from acetyl-CoA, which is two carbons shorter than before entering the cycle (Figure 2). The cleaved acyl-CoA then enters the Krebs cycle/citric acid cycle and subsequently the electron transport chain (ETC) within the mitochondria (Bartlett, Eaton 2004).

2.3.2 The mitochondrial electron transport chain

Oxidative phosphorylation generates ATP from the energy released by the oxidation of nutrients. OXPHOS is also known as the respiratory chain or electron transport chain. It consists of five different complexes: Complex I, II, III, IV and V that reside in the inner membrane of the mitochondria (Figure 3) (Yip et al. 2011). The electrons from NADH enter at complex I (NADH-dehydrogenase then converts NADH back to NAD⁺ by transferring electrons onwards to ubiquinone). At complex III (ubiquinol-cytochrome reductase) electrons are transferred to cytochrome c that is oxidized by complex IV (cytochrome c oxidase). Then electrons are finally transferred to molecular oxygen to produce water. Electrons from FADH₂ enter the respiratory chain at complex II (succinate dehydrogenase) that transfers them to ubiquinone. Pumping protons across the membrane into the inter membrane space is performed by the complexes I, III, and IV in an energy releasing process by electron transfer (Yip et al. 2011). Mitchell proposed this as a proton motive force that is defined as the combination of the proton concentration gradient (ΔpH) and membrane electrochemical gradient ($(\Delta \Psi_m)$) (Mitchell 1961). This is used by complex V that phosphorylates ADP into ATP to form the high energy entity (Boyer et al. 1973).

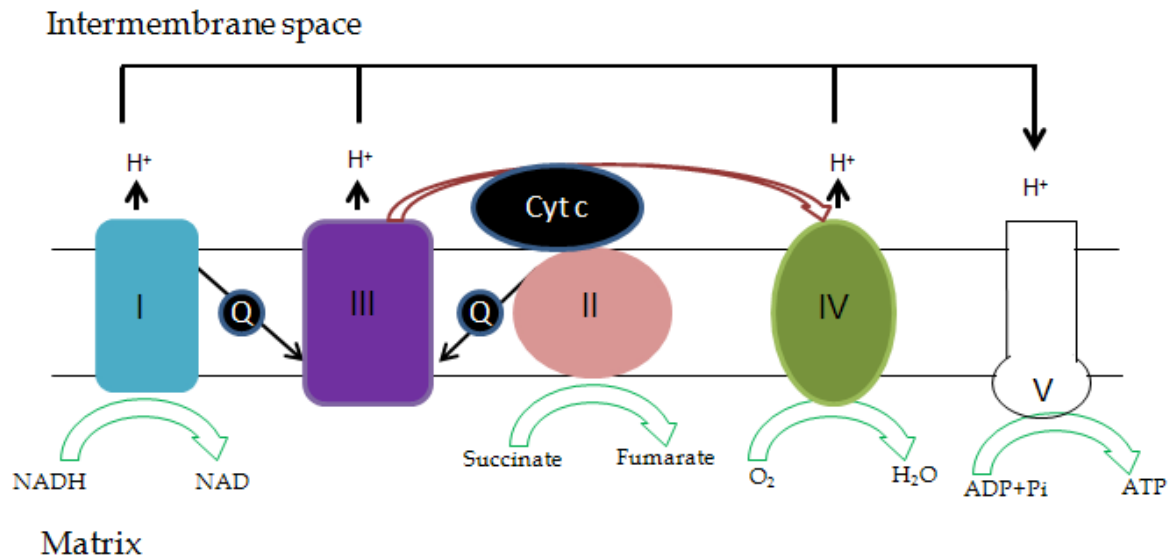


Figure 3 - Schematic representations of the mitochondrial electron transport chain and ATP synthesis. The electron transport chain consists of four complexes: **complex I**, **complex II**, **complex III**, **complex IV**, and **complex V**. Redox reactions are indicated below each complex and the direction of proton flow. Cytochrome *c* and coenzyme *Q* are located in the IMM and acts as an electron shuttles.

2.3.3 Other functions of mitochondria

Mitochondria actively participate in several other processes such as iron-sulphur cluster formation, haeme synthesis and steroid synthesis. Mitochondria are also involved in apoptosis and iron-copper metabolism (Michel et al. 1998).

2.4 Mitochondrial biogenesis

2.4.1 Mitochondrial DNA

Human mitochondria have their own genetic information that exists in a single circular mitochondrial DNA (mtDNA) molecule (Nass et al. 1963). Interestingly, mitochondria are inherited exclusively through the maternal lineage, whereas the paternal mitochondria are actively degraded during fertilization (Al Rawi et al. 2011; Sato et al.

2011). Human mtDNA is associated with the IMM and is organized in nucleoprotein complexes, known as nucleoids, which contain 2-8 mtDNA copies each (Holt et al. 2007). The copy number of mtDNA in human cells varies between 100-10000 copies. Generally, the mtDNA population is homoplasmic: only one mtDNA type exists in the organism whereas in heteroplasmy: two or more different mtDNA population exists in the same cell. The genetic code of mitochondria is distinct from the universal genetic code. The human mitochondrial genome is a 16.6 kb double stranded circular DNA molecule comprising of a heavy strand (H) and a light strand (L). The whole genome encodes only 37 genes: 22 transfer RNA's, 2 ribosomal RNA's and 13 subunits of the mitochondrial respiratory chain complex (Anderson et al. 1981).

2.4.2 Mitochondrial protein import

In eukaryotic organisms about 10% to 15% of the nuclear genes encode mitochondrial proteins (Neupert & Herrmann 2007). These are synthesized on cytosolic ribosomes, which are then transported into the mitochondria, a process that is termed mitochondrial protein import (Neupert & Herrmann 2007). The mitochondrial proteins are generally identified by an N-terminal positively charged presequence that is capable of forming a basic, amphipathic α -helix (Baker et al. 2007). All these mitochondrial proteins vary in function in a number of ways, some of which are in an unfolded conformation and which are associated with chaperones (Neupert & Herrmann 2007).

The crucial processes of membrane translocation and sorting of mitochondrial proteins into different mitochondrial compartments are ensured by a complex protein-import machinery that span the OMM and IMM (Baker et al. 2007 and Chacinska et al. 2009). The translocase of the outer membrane (TOM) is the universal entry gate for all precursor proteins that are imported into mitochondria (Baker et al. 2007). The components of the TOM complex include the receptors Tom20 and Tom70 and the translocation channel Tom40.

The proteins, which are targeted at the matrix, are sorted by the translocase of the inner membrane (TIM) that contains TIM23 as the central unit (TIM23 complex) (Baker et al. 2007). The import of proteins through the IMM is driven by the electrochemical potential ($\Delta\Psi$) and the chaperone mitochondrial Heat shock protein 70 (mtHsp70) (Young et al. 2003).

2.4.2.1 Chaperones

Molecular chaperones are a group of proteins that help in the efficient folding of 3D native structures in the cell (Frydmann 2001; Bukau et al. 2006; Hartl et al. 2011). One of the major important classes of chaperones is the Hsp70. The function and homeostasis of mitochondria has been dependent on mtHsp70 chaperones. The Hsp70 chaperone is highly homologous to bacterial DnaK chaperone protein (Craig et al. 1987). It exists in the mitochondrial matrix and mediates two different functions: folding of proteins and translocation of channel TIM23 (Endo & Yamano 2009; Mokranjac & Neupert 2010; Vander laan et al. 2010).

2.4.2.2 Role of TIM23 complex in mitochondrial membrane

TIM23 consists of membrane part that is tightly integrated into the membrane along with the import motor. The membrane part is made up of Tim23 and Tim 17 which are involved in forming the protein channel whereas Tim50 plays a role in the transfer of preproteins from the TOM to the TIM23 complex. (Mokranjac et al. 2003) The mitochondrial import motor is attached to the membrane part at the matrix face of the inner membrane. Three constituents TIM44, mtHsp70 and Mgel have been described. TIM44 is peripheral membrane protein involves with the segments of preproteins. TIM44 recruits mtHsp70 to binds to unfolded chains as they enter matrix followed by TIM44 release (Mokranjac et al. 2003). These reactions are performed by the hydrolysis of ATP bound to mtHsp70. Later Mokranjac and colleagues has identified TIM14 as an important part of TIM23 (Mokranjac et al. 2003). Tim14 is a member of the DnaJ protein family which consists of J – domain that faces the matrix space and a single transmembrane anchor in the inner membrane. Tim14 interacts with TIM44 and mtHSP70 in an ATP-dependent manner and has a effect on the interaction of mtHsp70 with TIM44. A mutation in the Tim14 will stop the function of J domain (Mokranjac et al. 2003).

2.4.2.3 Role of Cardiolipin in mitochondrial membrane

Cell organelles function is based on the membrane lipid composition. Cardiolipin (CL) present in all mammalian cells containing mitochondria. Cardiolipin is also termed as diphosphoglycerolipid. CL is localized in the inner mitochondrial membranes which is an important phospholipid. It plays a role in the function of mitochondrial biogenesis

and required for stability of several protein complexes (Richter-Dennerlein et al. 2014). CL biosynthetic pathway involves the enzyme activity of the protein encoded by the Taffazin gene (TAZ). TAZ play a key role in the cardiolipin remodelling in otherwords phospholipid acylation that includes the formation of monolyso-CL and subsequent acylation by TAZ. Defects or mutations in the acyltransferase tafazzin (TAZ) cause cardiomyopathy in Barth syndrome which is similar to DCMA syndrome. (Richter-Dennerlein et al. 2014).

2.5 Mitochondrial diseases

2.5.1 Clinical manifestations of mitochondrial diseases

Mitochondrial diseases are a heterogeneous group of disorders and have multisystem symptoms that cannot be described by a single diagnosis. Some mitochondrial disorders are caused by mutations in the mtDNA, whereas others are caused by mutations in the nuclear genome that expresses more than 1000 proteins that are subsequently imported and used in the mitochondria. Mitochondrial DNA is inherited maternally (Gillis et al. 2002) and many mtDNA point mutations contribute to a number of neurological diseases for example Pearson syndrome and respiratory chain deficiency. Most of the known mitochondrial disorders are considered to be neuromuscular diseases or mitochondrial encephalomyelopathies. The most prominent neuromuscular manifestations are seizures, strokes, dementia, ataxia, optic neuropathy, peripheral neuropathy, myopathy, retinopathy, and sensorineural hearing loss. (Gillis et al. 2002) However 33% of the mitochondrial disorder patients have non- neuromuscular symptoms (Munnich et al. 1992, 1996). Most of the mitochondrial disorders are characterized by multiorgan involvement and affect any of the energy-demanding organs including: the brain, heart, liver, and skeletal muscle (Kerr 1997). The age of the patient upon disease onset can vary from neonate to adult. In neonates the clinical features observed are hypotonia, muscle weakness, lethargy, feeding and respiratory difficulties, failure to thrive, psychomotor delay, seizures, and vomiting (Sue et al. 1999).

Clinicians should be well experienced and have a thorough knowledge of the symptoms and signs that the patient could exhibit in order to diagnose mitochondrial disorders in patients. First, the clinician should know the medical history of the family and the relatives of the patient and for the biological parents, if they are consanguineously related. This is especially vital if the biological parents who have also experienced similar symptoms and signs earlier. Second, if the affected family did have a similar case previously/historically, the clinician should study their pedigree inheritance. Third, the

clinician should suspect the occurrence of a particular mitochondrial disease upon reappearance of similar symptoms (Suomalainen 2011).

Diagnostic tests for mitochondrial fatty acid β -oxidation disorders include the following: determining the plasma acyl carnitine profile, total and free carnitine levels, urine organic acid determination, molecular genetic analyses and measurement of the cerebrospinal fluid parameters (Gillis et al. 2002). Usually urinary organic acids are found to be abnormal, as they have increased levels of 3-methylglutaconic acid, 2-methylsuccinate. Further histological (skeletal muscle and liver biopsy) and respiratory enzyme analyses are conducted. Tandem mass spectrometry is the most useful method to identify acylcarnitine species from blood spots either as a part of neonatal screening or otherwise for diagnosis of mitochondrial fatty acid oxidation disorders (Gillis et al. 2002).

2.5.2 Mitochondrial β -oxidation defects of FAs

In general mitochondrial β -oxidation defects of FAs have an early-onset, sometimes identified even in the neonatal and infant stages and are inherited in an autosomal recessive fashion. However, there may be great heterogeneity within families and individuals and an important influence of environmental and modifying genetic factors (Vockely & Whiteman 2002). The first genetic defect of FAO in human was identified, as a disorder of the skeletal muscle that presented with exercise induced rhabdomyolysis and myoglobinuria in 1973 (Dimauro et al. 1973). A case was reported with hepatic presentations later on during the early 1980s, which was eventually diagnosed as medium-chain acyl CoA dehydrogenase (ACADM) deficiency/impairment (Stanley et al. 1983). The main fatty acid oxidation (FAO) defects are listed in Table 1 and CPT1A, LCHAD and DCMA defects are described briefly below.

2.5.3 CPT proteins and carnitine uptake machinery

The CPT system constitutes two separate proteins that are located in the outer (CPT1) and inner (CPT2) mitochondrial membranes. CPT1 belongs to a protein family that includes several other acyl transferases and acyltransferases that do not use carnitine as second substrate. Three tissue specific isoforms of CPT 1 are known to exist: liver (CPT1A), muscle (CPT1B) and brain (CPT1C). The CPT1A protein consists of 773 amino acids. The *CPT1A* gene is localized in the 11q13.1-q13.5 range in the human chromosome. The protein's structural model has been proposed based on the carnitine acetyltransferase crystal structure (Morillas et al. 2004)

The age of the CPT1 defect onset usually occurs from the first month of life to 18 months. Onset is usually initiated with fasting or viral illnesses. The patients usually present with hepatomegaly and an altered mental status. In such cases, biochemical tests usually reveal nonketotic hypoglycaemia, mild hyperammonaemia, elevated liver functioning and, elevated free FAs (Longo et al. 2006). Increased plasma carnitine levels are usually observed in this disease. Urine organic acid analysis shows high levels of C12 dicarboxylic acid and the presence of 3-hydroxyglutaric acids. Diagnosis is suspected from the increased levels of free and short chain acylcarnitines, with low levels of long chain acylcarnitine. Confirmation of disease can be obtained by measuring the activity of the CPT1 enzyme usually from fibroblasts. The enzyme activity is usually reduced to 5-20% of normal levels. The ratio between free carnitine (C0) and the sum of palmitoylcarnitine and stearoylcarnitine (C16+ C18) is elevated in patients with CPT1A deficiency and cause shuttle defective. (Longo et al. 2006). Further DNA studies will reveal the specific mutations causing disease.

Table 1 – Defects in fatty acid transport and enzymes involved in fatty acid β -oxidation and defects in protein import

Deficiency	Gene Symbol	OMIM	Function	Clinical manifestations	Onset of disease	Location of enzyme in mitochondria	References
Carnitine palmitoyltransferase deficiency 1	<i>CPT1</i>	600528	Transport of fatty acids	Fatty liver, nonketotic hypoglycaemia	Neonatal	Plasma membrane	Ijlst et al. 1998
Carnitine-acylcarnitine translocase	<i>CACT/SLC25A20</i>	613698	exchange acycarnitines for the carnitine molecule	Liver failure, hypertrophic cardiomyopathy and septal heart defects	Neonatal	Plasma membrane	Huizing et al. 1998
Very long chain acylCoA dehydrogenase deficiency	<i>ACADVL</i>	609575	Catalyze in mitochondrial palmitoyl-CoA dehydrogenation	dilated cardiomyopathy skeletal myopathy, hypoketotic hypoglycaemia, Reye-like disease	Neonatal -Infant	Membrane	Aoyama et al. 1995
Medium chain acyl CoA dehydrogenase deficiency	<i>ACADM</i>	607008	Catalyzes the initial reaction in β -oxidation of C4-C12 straight chain acyl CoA's	hypoketotic hypoglycaemia, common infections	Neonatal	Matrix	Matsubara et al. 1990
Short chain acyl CoA dehydrogenase deficiency	<i>ACADS</i>	606885	Detoxification of metabolic products	Muscle hypotonia and developmental delay	Neonatal	Matrix	Naito et al. 1989
Isolated Long chain ³ hydroxyacyl-CoA dehydrogenase deficiency	<i>HADHA</i>	609016	Catalyzes long-chain fatty acids	Acute hepatic failure, cardiomyopathy, skeletal myopathy, retinal pigmentary changes and neuropathy	Early-onset	Matrix	Wanders et al. 1989
Mitochondrial trifunctional protein deficiency	<i>HADHA/HADHB</i>	609015	Catalyzes long chain fatty acids	Cardiomyopathy, muscular hypotonia,	Early-onset	Matrix	Wanders et al. 1992
DCMA or 3-methylglutaconic aciduria, type V	<i>DNAJC19</i>	610198	Mitochondrial protein import motor-similar to TIM14	Dilated cardiomyopathy, ataxia	Early-onset	Inner mitochondrial membrane	Davey et al. 2006

2.5.4 Long chain 3-hydroxyacyl-CoA dehydrogenase deficiency

Long chain 3-hydroxyacyl-CoA dehydrogenase deficiency (LCHADD) was initially described in 1989, and by 1992 at least 10 patients had been identified, but the carrier frequency of LCHAD deficiency was not known. Existence of the trifunctional protein complex was proven in 1992 (Wanders et al. 1989). LCHADD (OMIM#609016) is caused by a point mutation and it lies in the alpha subunit of the mitochondrial trifunctional protein, encoded by *HADHA*. The most common disease causing variant in LCHADD is c.1528 G>C single nucleotide substitution leading to the amino acid change, p.Glu510Gln, in *HADHA* (Ijlst et al. 1994). This results in hypoketotic hypoglycaemia during times of fasting, illness and or/ prolonged exercise. Of all the FA β O disorders, LCHADD is associated with the greatest number of severe complications. The initial cases of isolated LCHADD diagnosed, presented with severe liver pathology, cardiomyopathy, rhabdomyolysis and/or myoglobinuria and sudden death. Out of 30 cases, of isolated LCHAD deficiency 50% had retinopathy and peripheral neuropathy. Pigmentary changes in the retina were observed by the age of 2 years in around 50% of individuals with LCHADD. A high level of long-chain hydroxylated fatty acid 3-hydroxyacylcarnitine is observed in the patient's plasma, (Wanders et al. 1989).

The p.Glu510Gln variant that caused LCHADD was diagnosed in 15 individuals in 1998. Patients with LCHADD were observed to develop granulation with pigment clumping in the macula of the retina, which was visible as early as four months of age. Although many patients had died before two years of age, more than 50% of them had abnormal fundus examinations prior to death. Tyni and colleagues proposed four stages of LCHADD retinopathy based on these aforementioned data (Tyni et al. 1998a).

- Stage 1 normal retinal function and a hypopigmented fundus are observed.
- Stage 2 is distinguished by the appearance of pigment clumping in the fovea in addition to progressive retinal dysfunction as measured by electroretinogram (ERG). However, despite the diminution in ERG amplitudes, age-appropriate performance and visual acuity remain intact.
- Stage 3, central pigmentation disappears as chorioretinal atrophy that lead to notable macular pallor and pigmentary changes, which migrate towards the periphery. ERG readings continue to decline with markedly reduced amplitudes and prolonged implicit times, or become unrecordable. At this stage the patient, often reports loss of night vision.
- Stage 4, at the end-stage, the posterior pole of the eye loses all photoreceptors, and most of the choroidal vessels and macular (central) vision are also lost. Morphology reminiscent of the previous stages can be seen spreading outward to the peripheral retina as progression continues in stage 4 (see Figure 4).

Histopathological examination of one of the LCHADD patient's eyes at 7 months of age and who died at 14 months of age revealed macrophage infiltration of the retinal pigment epithelial (RPE) layer and the evidence of RPE cell death. This suggests that disruption or dysfunction of the RPE layer eventually leads to the loss of the photoreceptors (Tyni et al.1998b).

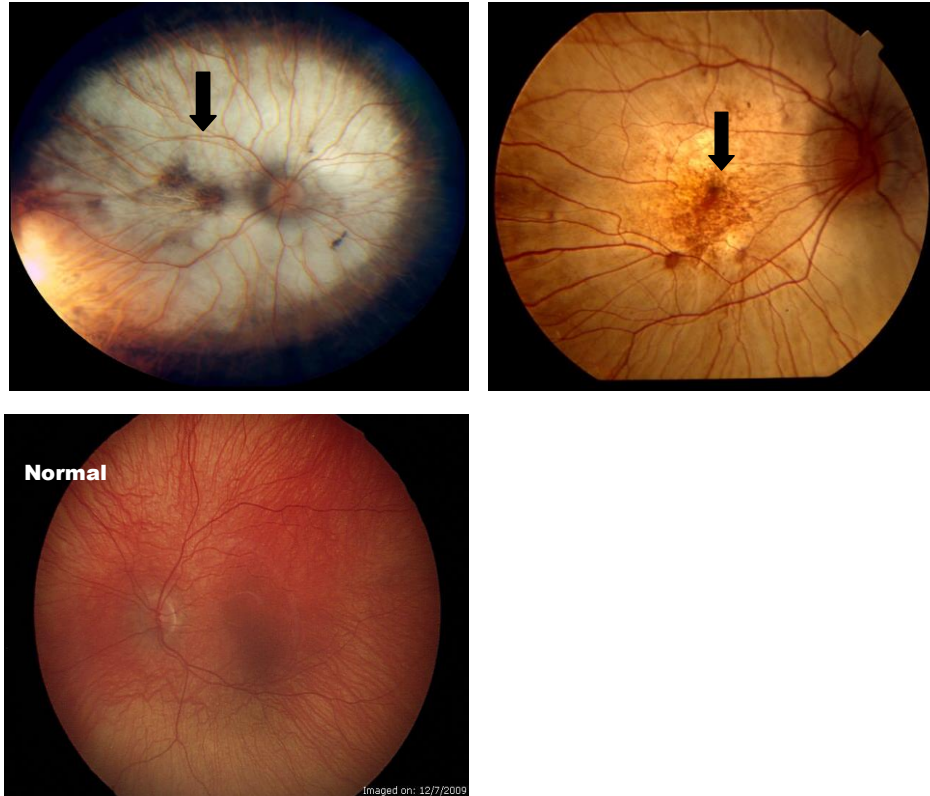


Figure 4 – Photographs showing the fundus of LCHADD patients at stage 4 and Below-Normal fundus image (Permission received from ethical committee and patients). Pigment clumping and granulation can be observed in fundus image (Arrows).

2.5.5 Dilated cardiomyopathy with ataxia syndrome

Dilated cardiomyopathy with ataxia (DCMA) syndrome is a novel autosomal recessive disorder. It is an inherited condition with manifestations that include heart defects, movement difficulties, and other characteristics that affect multiple body systems. Usually DCMA develops in infancy to early childhood with dilated cardiomyopathy in which the heart enlarges and weakens thereby adversely affecting its blood pumping efficiency. This syndrome is associated with increased levels of 3-methylglutaconic acid and 3-methylglutaric acid in urine. So far 30 cases have been reported in the Dariusleut Hutterite population in Canada (Sparkes et al. 2007). This syndrome shares resemblance to Barth syndrome (type II 3-methylglutaconic aciduria), which is an X-linked disorder caused by mutations in the tafazzin (*TAZ*) gene. It has been found that variants in the

DNAJC19 (DnaJ (Hsp40 homolog, subfamily C, member 19) gene lead to DCMA syndrome. The *DNAJC19* gene in humans encodes the mitochondrial import inner membrane translocase subunit TIM14 enzyme. The disease-causing variant identified in the Canadian Dariusleut Hutterite population causes aberrant splicing and results in the loss of full length *DNAJC19* transcript. Proteins containing the DNAJ domain are typically involved in molecular chaperone systems of the Hsp70/Hsp40 type. These chaperone systems aid in the folding and assembly of newly synthesized proteins (Davey et al. 2006). Chaperones prevent abnormal protein folding and their subsequent aggregation during times of stress (Davey et al. 2006).

2.6 Pluripotent stem cells

Pluripotency is defined as the capacity of a cell to develop into any cell type that is found in embryonic and adult organisms (except extra-embryonic organs including the placenta and the umbilical cord). Stem cells in general also have a massive capacity for self-renewal and therefore these cells have the potential to play a major role in regenerative medicine and other cell-based therapies. Human embryonic stem cells (hESCs) are derived from the excess embryos created during the *in vitro* fertilization process (Thomson et al. 1998), whereas human induced pluripotent stem cells (hiPSCs) are generated by reprogramming of adult somatic cells back into the pluripotent state using the “Yamanaka” embryonic transcription factors (Takahashi et al. 2007). A team lead by Takahashi and Yamanaka in 2006 discovered that retroviral vector based expression of four transcription factors: Oct4, Sox2, Klf4 and c-Myc can induce embryonic stem cell like cells from somatic cells (Figure 5). These cells showed similar characteristic features of pluripotency as ESCs and therefore they were called iPSCs.

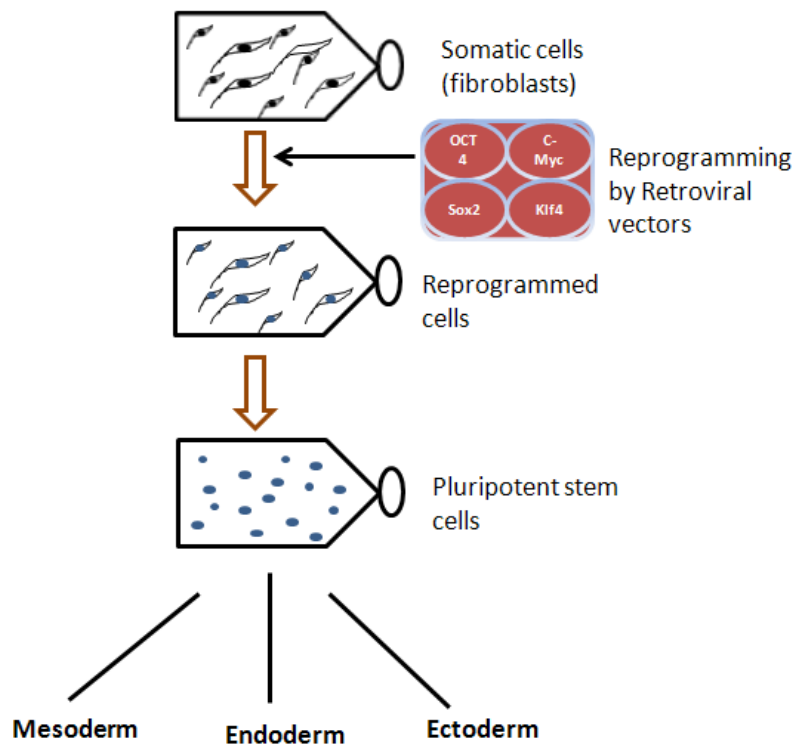


Figure 5 - Method of generating induced pluripotent stem cells (iPSC) by retroviral vectors.

2.6.1 Characteristics of pluripotent stem cells

Molecular and functional characterization is necessary to distinguish fully reprogrammed iPSCs from partially reprogrammed cells (Takahashi et al. 2007). Generally human pluripotent stem cell (hPSC) cultures consist of several cell types that range from self-renewing or undifferentiated stem cells to incipient lineage-biased cells. Pluripotency is a process that involves multiple genes and pathways. It has been a major challenge in understanding the underlying mechanisms that control the status of the hPSC (Brivanlou and Darnell 2002). Morphologically, the hPSC have high nucleus to cytoplasmic ratio, prominent nucleoli and distinct epithelial cell-like colony morphology with sharp edges and a round shape (Thomson et al. 1998; Reubinoff et al. 2000). During the process of differentiation cellular morphology changes occur rapidly.

2.6.1.1 Markers to identify pluripotent stem cells

A set of markers are usually used to monitor the differentiation status of hPSCs (Adewumi et al. 2007). Conventionally the various markers used include Oct4 (octamer-binding transcription factor 4), SOX2, tissue non-specific alkaline phosphatase markers Tra 2-49 and Tra 2-54, a high molecular antigen GCTM2, cell surface glycoprotein epitopes Tra-1-60 and Tra-1-81, glycosphingolipid antigen H type 1 and the stage – specific embryonic antigens SSEA-3 and SSEA-4 (Adewumi et al. 2007, Takahashi et al. 2007). Nanog was identified as a pluripotency factor more than ten years after Oct4 and Sox2 were identified (Chambers et al. 2003; Mitsui et al. 2003). Thus, the discovery of iPSCs have helped us in gaining a better understanding of the mechanism of key transcription factors that are required for early development.

Earlier work showed that specific transcription factors Oct4 (also known as Pou5f), SOX2, a member of the SRY-related high mobility group-box (SOX) family and Nanog, a homeobox protein, are key regulators of early development (Scholer et al. 1989; Scholer et al. 1990a; Scholer et al. 1990b; Nichols et al. 1998). This OCT4-SOX2-NANOG-triad is central to the transcription regulatory hierarchy that specifies the identity of hPSCs. These transcription factors regulate several hundreds of target genes in the pluripotent cells, which indicate their crucial role in pluripotency. However, the expression of this triad does not guarantee pluripotency *per se* because, factors such as Kruppel-like factor (Klf4) are also vital to achieve pluripotency (Scholert et al. 1990b; Chambers et al. 2003; Lee et al. 2004). Klf4 plays a critical role in the process of proliferation, differentiation and apoptosis (Garrett-Sinha et al. 1996; Shields et al. 1996; Rowland et al. 2005).

2.6.2 In vitro differentiation of human pluripotent stem cells to retinal pigment epithelial cells

2.6.2.1 The human eye

The eye is like a camera that can self-focus, adjust for light intensity, and convert light into electrical impulses that can be interpreted by the brain. The human eye is located in a bony orbit and is connected to the brain by the optic nerve. The eyeball protects and facilitates the functions of the photoreceptive retina, and inner layer of the eye ball (Kierszenbaum 2002). The human eye-ball is roughly spherical with a diameter of about 24 mm. The anterior pole of the eye-ball is the centre of the cornea. The posterior pole is located between the optic disk and the fovea, in a shallow depression in the retina (Figure 6) (Kierszenbaum 2002).

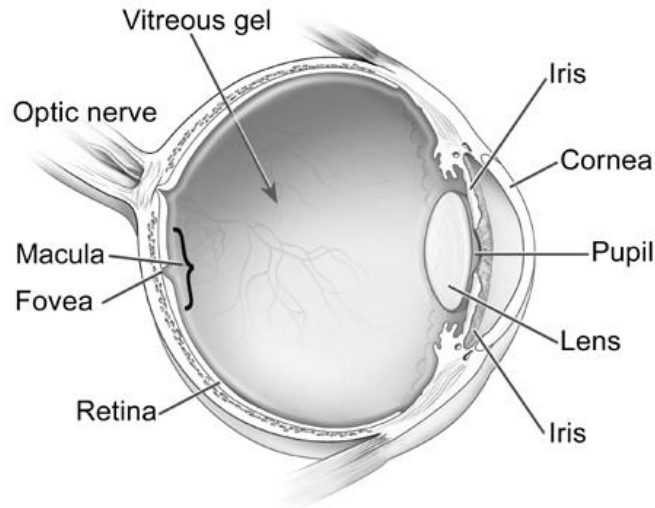


Figure 6 – *Diagram of the Eye-* (Courtesy: National Eye Institute, National Institutes of Health).

2.6.2.2 Structure of the posterior segment of the human eye

The outer layer of the posterior segment is known as the sclera that functions as the main supporting layer of the posterior part of the eye due to its rigid and collagenous structure. It is a brachytrophic structure with a low metabolic rate. The choroid is a thin, richly vascularised layer between the sclera and the retinal pigment epithelium (RPE). The choroid consists of three layers: (a) Bruch's membrane, (b) the choriocapillaris, and (c) the stroma. The choriocapillaris is a dense network of wide, fenestrated capillaries, arranged as one single layer. The stroma contains larger choroidal vessels and melanocytes. Blood is supplied to the choroid by the short and long posterior ciliary arteries. The main function of the choroid is to nourish the outer third of the retina, particularly the photoreceptor cells (Figure 7) (Kierszenbaum 2002).

2.6.2.3 Cell layers of the retina

In addition to the RPE, the inner neurosensory retinal layer can be further divided into nine layers, including the photoreceptor layer, ganglion layer, bipolar layer and the nerve fibre layer all of which are composed of cell bodies and processes of its neuronal and glial cells (Figure 7). The inner limiting membrane consists of foot plates of the Müller's radial glial cells that lie between the neuroretina and the vitreous body. Müller's cells extend a supportive and nutritive function. The ganglion cell layer comprises a single layer of ganglion cells in the peripheral retina and up to 10 layers in the fovea. The inner nuclear layer contains three main types of interneurons: bipolar, amacrine, and Müller cells. In the outer nuclear layer, the cone nuclei form a monolayer immediately below

the outer limiting membrane, the rest of the nuclei belong to the rods. The inner and outer plexiform layers consist of the synapses of these five retinal layers. The inner and outer segments of the rods and cones form the photoreceptor layer. The organelles (mitochondria, ribosomes) that are involved in the highly active energy production and in the protein synthesis of the photoreceptor cells are situated in their inner segments. Cones predominate in the central retina and are present exclusively in the foveal centre, whereas rods predominate in the periphery (Kierszenbaum 2002).

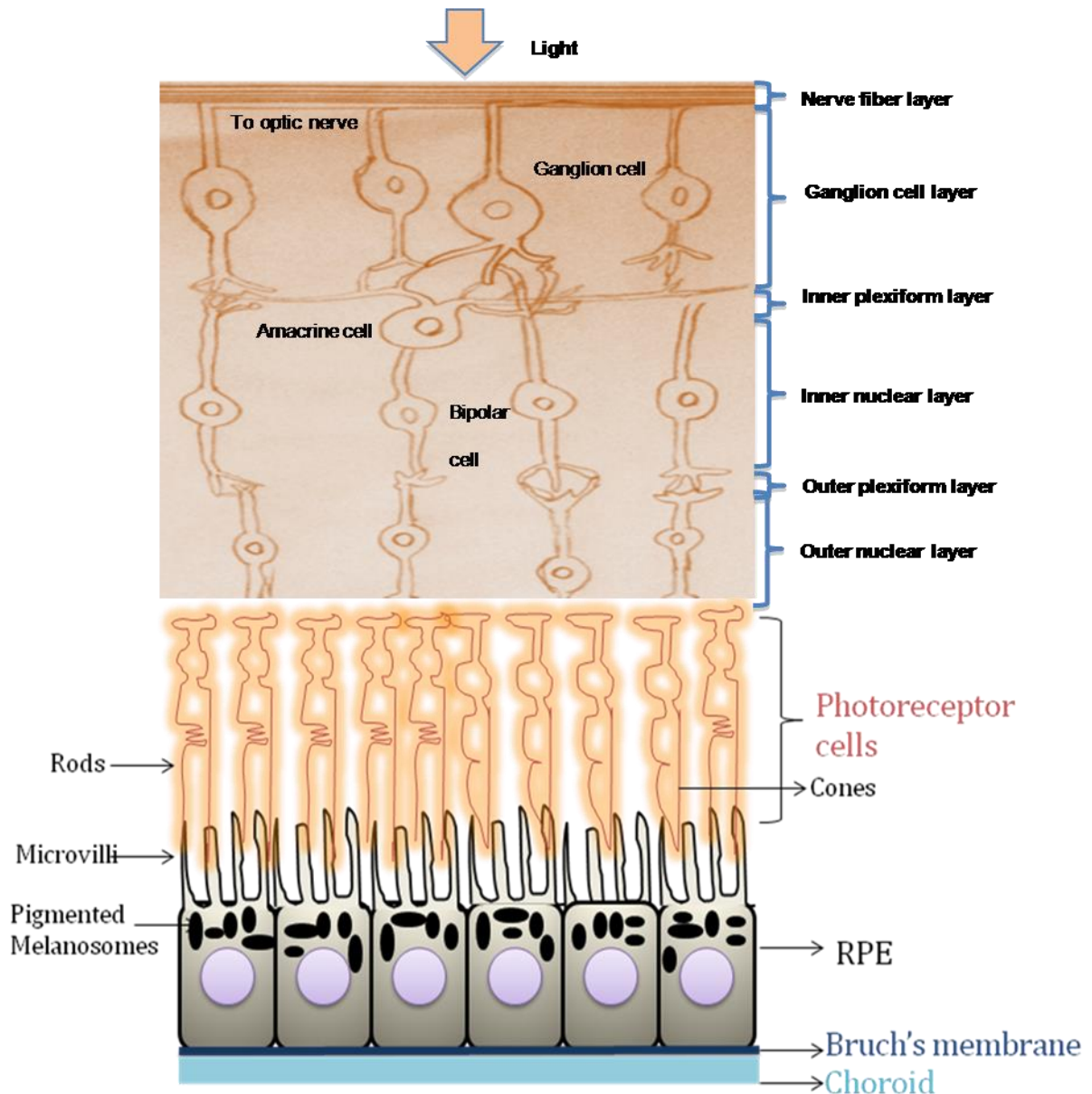


Figure 7 – Cell layers of the retina & Structure of RPE showing the photoreceptor cell arrangement (Modified picture from Bharti et al. 2011).

2.6.2.4 The Retinal pigment epithelium (RPE)

The RPE is a monolayer of pigmented, polarized and highly specialized epithelium. It constitutes of hexagonal pigmented epithelial cells that are attached to the Bruch's membrane. The apical membrane of the RPE cells faces the photoreceptor and the apical RPE microvilli surround the photoreceptor outer segments (POS). The basolateral membrane of the RPE faces the Bruch's membrane, a specialized basement membrane of the RPE (Figure 7). These cells also play a role in the formation of the outer blood-retinal barrier participating in the control of ions, nutrients, and metabolites that are transported between the photoreceptors and choroid (Strauss 2005). The RPE also plays a key role in the visual cycle of the retina by re-isomerizing all trans-retinal to 11-cis-retinal *via* a number of enzymes including the 65kDa retinal pigment epithelium-specific isomerase (RPE65). The RPE cells are metabolically active; they phagocytose and can also degrade the photoreceptor outer segments damaged by light (Strauss 2005, Bharti et al. 2011).

2.6.2.5 The development of RPE cells

The RPE cells exhibit cobblestone morphology and *in vitro*, grow as a single monolayer arranged in a hexagonal mosaic pattern and pigmentation due to the presence of melanosomes that are stored in melanin pigments (Figure 7) (Maminishkis et al. 2006). Both the RPE and the neural plate are derived from the anterior neural plate during embryogenesis. Stage two bilateral optic primordia are formed from the neural plate to give rise to the both the eyes at maturation (Zaghloul et al. 2005). The neural plate converts into the neural tube, further the optic vessels are formed by the evagination of optic primordia from the diencephalon. Later the optic vessels transform into optic cups, with two different layers: inner layer and outer layer form. The inner layer gives rise to neural retina that contains a whole range of cells right from the cone and rod photoreceptors, in addition others such as Müller, glial, ganglion cells, horizontal, bipolar and amacrine cells. The outer layer forms the RPE (Zaghloul et al. 2005).

A number of transcription factors are required for eye development: Orthodenticle homeobox-2 (OTX2), paired box protein-6 (PAX6), retina and anterior neural fold homeobox (RAX), and SIX homeobox 3 (SIX3) (Zuber et al. 2003). The presumptive RPE faces the periocular mesenchyme while the prospective neural retina faces the surface ectoderm during the stage of optic vesicle formation (Martinez-Morales et al. 2004). FGF is expressed by the ectoderm that faces the prospective neural retina and is responsible for the induction of early retinal transcription factors, such as Chx10 in the inner layer (Nguyen & Arnheiter 2000). The extraocular mesenchyme secretes activin A

whilst the optic vesicle expresses activin receptor suggesting that TGF β signalling participates in establishing RPE identity (Martinez-Morales et al. 2004). The transcription factors that are proven to be essential for RPE specification are: Microphthalmia-associated transcription factor (MITF), OTX1/OTX2, and PAX6.

MITF is a transcription factor of the basic helix-loop-helix and leucine zipper family (bHLH_LZ). The fundamental function of MITF involves the development of melanin-producing cells, including the neural-crest-derived melanocytes and the RPE (Martinez-Morales et al. 2004, Goding 2000). In fact MITF is vital for the activation of expression of pigment specific genes such as tyrosinase, tyrosinase related proteins TRP1 and TRP-2 and QNR71 (Fuhrmann et al. 2000). MITF operates as a target and also as a nuclear mediator of Wnt signalling in melanocyte development. Further, the Wnt signalling cascade in the RPE may help in maintaining MITF expression (Martinez-Morales et al. 2004). The important feature of MITF is its gene structure and its isoforms. MITF consists of five isoforms with distinct amino-termini: MITF A, MITF B, MITF C, MITF H, MITF M. MITF M is important for melanocyte differentiation whereas other isoforms are responsible for RPE differentiation and normal eye development (Shibahara et al. 2001).

OTX1 and OTX2 are transcription factors that each contains a homeodomain region. The expressions of OTX genes initially occur in the entire optic vesicle and are later restricted to the presumptive RPE during optic cup formation (Martinez-Morales et al. 2004). PAX2 and PAX6 are the two members of the paired box family of transcription factors expressed in the optic territory during development. PAX6 is distributed mainly in the entire optic vesicle and the early optic cup (Walther et al. 1991). During the late optic cup stage, the expression of PAX6, however, disappears from the developing RPE (Grindley et al. 1995).

The initial network for driving the RPE differentiation process comprises PAX6 and Wnt signalling, which in combination, switches on the expression of MITF. This switching occurs in cooperation with OTX proteins (Martinez-Morales et al. 2004). Other molecules such as Bone Morphogenetic factor (BMP) and Hedgehog (Hh) families and the cell cycle regulators are involved in the differentiation of RPE (Martinez-Morales et al. 2004).

2.6.2.6 RPE differentiation procedures *in vitro*

Klimanskaya and co-workers were the first to demonstrate that hESCs can spontaneously differentiate into RPE-like cells when they are overgrown on mouse embryonic feeder cells (Klimanskaya et al. 2004). The same authors have also reported that hESC can also differentiate without feeder cells as embryonic bodies in standard

hESC culture medium (KO-SR) but in the absence of bFGF. The differentiations of hESC and hiPSC have been based on either adherent overgrowth on feeder cells or embryoid body (EB) or neurosphere/rosette formation in suspension.

It has been noticed that pigmentation usually appears within eight weeks of culture. After sufficient pigmentation is achieved, pigmented areas are manually selected and seeded onto the extracellular matrix (ECM) protein coatings for RPE cell enrichment. The differentiations are generally inefficient and slow, thus it may take months to acquire enough pigment cells for enrichment. After replating, the hiPSC-RPE cells lose their pigmentation and acquire fibroblast-like morphology; they also readily proliferate to confluence and re-differentiate to the RPE cell phenotype (Klimanskaya et al. 2004). The transdifferentiation resembles the epithelial-mesenchymal-transition (EMT) process that is also described for the isolated native RPE cells upon loss of cell-cell contacts (Tamiya et al. 2010). The spontaneous and embryoid differentiation methods lead to the generation of multiple cell types and the sequence of events that leads to the RPE cell formation is still unclear. It is generally accepted that in the absence of inductive cues, the ESCs 'choose' the neural differentiation pathway as a "default" pathway (Smukler et al. 2006). It is possible that the earliest step is a default differentiating step of the neural lineage commitment of neuroectodermal cells or retinal progenitors and the further specification to RPE cells is driven by the surrounding cells in the EBs or the feeder cells (Klimanskaya et al. 2004).

2.6.2.7 Characterization of human pluripotent stem cell-derived retinal epithelial cells

Human iPSC-RPE cells show typical pigmentation and morphology quite similar to their native counterparts. Their genes express proteins that are specific for RPE cells, which include a number of transcription factors including: MITF, OTX2, the membrane associated proteins bestrophin, tight junction protein zona occludens (ZO-1), proteins involved in the retinal visual cycle, cellular retinaldehyde-binding protein (CRALBP) and RPE65, tyrosinase and premelanosome protein (PMEL). Tyrosinase and PMEL play a key role in pigment synthesis and finally proteins that are involved in phagocytosis such the integrin αV subunit and Mer Tyrosine Kinase (MERTK) (Lund et al. 2006; Vaajasaari et.al. 2008; Buchholz et.al. 2009; Carr et.al. 2009; Liao et al. 2010; Vugler et.al. 2008; Cho et.al. 2012). Additionally, human iPSC-RPE cells exhibit a gene expression signature similar to native RPE (Liao et.al. 2010; Lamba and Reh 2011) and dynamic regulation of specific miRNA subsets associated with the RPE differentiation process (Li WB et al. 2012). Mature hiPSC-RPE cells show polarized apical and basal features and barrier function with increasing transepithelial electric resistance (TER) and impedance and decreasing permeability upon being measured by electrical impedance spectroscopy (Savolainen et al. 2011; Vaajasaari et al. 2011; Zhu et al. 2011; Onnela et al. 2012). Further, it has been demonstrated that polarized hPSC-RPE cells display localization of proteins e.g. apical localization of Na⁺/K⁺ ATPase and basolateral localization of bestrophin and polarized secretion of vascular endothelial growth factor

(VEGF) to the basolateral side (Kokkinaki et al. 2011; Vaajasaari et al. 2011). Polarized hESC-RPE cells also have prominent expression of pigment epithelium-derived factor (PEDF) in apical cytoplasm and an increased secretion of PEDF into the medium as compared with non-polarized culture (Zhu et al. 2011).

The functionality of hPSC-RPE cells is shown by their ability to phagocytose latex beads or more specifically photoreceptor outer segments (Klimanskaya et al. 2004; Buchholz et al. 2009; Liao et al. 2010) and also in rescuing visual function as observed in the royal college of surgeons (RCS) rat model (Lund et al. 2006; Vugler et al. 2008).

The hiPSC-RPE that shows regular physiological functions can be produced *in vitro* by well-established protocols. Interestingly, RPE cells that are derived from hiPSCs are the only cells that have met the high standards required for clinical trials. Initially, there was success in transplanting iPSC derived RPE cells in animals. Currently the work is very active with clinical trials for macular degeneration and retinitis pigmentosa disorders (Svendsen et al. 2013) but the drawback of using iPSC technology is tumour formation and host immune rejection of transplanted cells.

Recently, Schwartz and colleagues succeeded in the use of hESC-RPE cells in treating age related macular degradation and Stargard's dystrophy in a clinical setting (Schwartz 2012, 2014). After the transplantation of hESC-RPE cells into the patients' eyes, hyperproliferation, tumour formation, immune rejection or serious ocular problem were not observed, which in itself is a major success. Remarkably, the patients showed improved vision, which therefore suggest that the hESC-derived cells could provide a new alternative therapeutic procedure in treating disorders that require tissue replacement. (Schwartz et al. 2012, 2015). These may contribute to further success of iPSCs in medical therapies (Svendsen et al. 2013).

Reprogramming aids us exciting possibilities for studying and treating diseases. In future iPSC cells help us to provide unlimited cells and tissues for many patients with untreatable diseases. These models can also help us to test new drug therapeutics to treat diseases. Current reprogramming strategies include retroviral, lentiviral, adenoviral and plasmid transfections to provide reprogramming factor transgenes. A small molecule also helps in reprogramming transgenes and they also increase the efficiency of generating iPSC lines. (Gunaseeli et al. 2010)

Another emerging platform deals with iPSCs and genome editing technology to generate human disease models. This is possible using zinc finger nucleases (ZFN), which provide short template complementary sequences combined with integrases, or by adenoviral delivery. The use of ZFN genome editing tool, however is not straight forward, further it is labour intensive and quite expensive. Very recently, the enzyme transcription activator-like-effect or nucleases (TALENs) have been used. These are

inexpensive and have a good specificity but the frequency of recombination is low. Another genome editing tool, CRISPR/CAS systems seem to be very efficient at targeting iPSCs. Improving gene editing techniques by iPSCs might help to remove tumour formation in the long-run (Svendsen et al. 2013).

3 AIMS OF THE STUDY

The main objective of the research reported in this thesis was to study mitochondrial disorders and their pathogenesis. This thesis is based on studies relating: 1) First, detecting mitochondrial FA β O (fatty acid β - oxidation) and mitochondrial chaperone defects and also to carry out their molecular genetic analyses. 2) Second, introduce an *in vitro* cell model that helps to study the underlying pathogenesis of a retinopathy condition that expresses the prevalent variant p.Glu510Gln in LCHAD deficiency.

The aim of this thesis was planned based on the symptoms and biochemical analysis results obtained from the patient's blood and urine samples. The specific aims of this study were:

1. To find out the molecular genetic defects of FA β O from long term follow up of 6 patients with CPT1A deficiency diagnosed by biochemical analysis and to study the carrier frequency of the identified *CPT1A* p.Lys455Thr variant in the Finnish population
2. To analyse the clinical features of a rare mitochondrial chaperone disease and find out the molecular genetics of DCMA syndrome
3. To study the underlying pathogenesis of pigmentary retinopathy related to the prevalent (1 in 200) variant p.Glu510Gln in LCHAD deficiency

4 MATERIALS AND METHODS

The materials and methods used in this study are presented in detail in the original publications (I -III), which are referred to by their roman numerals and are briefly summarized in this thesis.

4.1 Ethics statement (I - III)

All the participating patients gave their signed informed consent and all the study protocols were approved by the Ethics committee of Helsinki University Central Hospital, Helsinki, Finland.

4.2 Subjects (I - III)

We studied the long-term follow up of six patients with CPT1A deficiency diagnosed by biochemical analyses and 150 healthy controls were also used to determine *CPT1A* carrier frequency. In addition, two patients with DNAJC19 deficiency and two patients with LCHAD deficiency, diagnosed by biochemical analyses and healthy controls were included.

4.3 DNA extraction (I - III)

Extraction of DNA was performed from blood and fibroblasts from patients and controls in studies I and II. In study III the DNA was extracted from undifferentiated hiPSC and patient's hiPSC derived RPE cells. The extractions were performed using the flexi gene DNA kit from QIAGEN™ (QIAGEN™). Concentrations of DNA were measured using Nanodrop at an absorbance of 260nm.

4.4 Protein extraction (I - II)

Protein isolation from fibroblasts of patients and controls (I, II) were done according to the method described by Prip-Buus (Prip-Buus et al. 2001) and protein quality was assessed by Lowry's assay. Mitochondrial protein extraction for blue native polyacrylamide gel-electrophoresis (BN-PAGE) from heart and skeletal muscle were extracted as described below. Crude mitochondria was prepared by homogenizing the tissue samples in ice cold HIM buffer (200mM Mannitol, 70mM sucrose, 10mM HEPES, 1 mM EGTA, pH 7.5 with KOH). Homogenates were centrifuged at 600 xg for

20 minutes at +4°C, the supernatant was transferred to a new tube and centrifugation repeated at 600 xg for 20 min. The supernatant was then centrifuged at 8000 xg at +4°C for 10 min, to get enriched mitochondria. The mitochondrial pellet was washed in ice-cold HIM buffer and the centrifugation step repeated. The samples for the BN-PAGE assay were then stored in 750mM aminocaproic acid and 5% serva blue G (Serva) in aliquots stored at -80°C.

4.5 Mutation Analysis (I - III)

We used PCR to amplify the exon regions of the control and patient DNA in order to identify variants in *CPT1A*, *DNAJC19* and *HADHA*. PCR was carried out with the high fidelity Phusion® DNA polymerase (Thermo Fisher, Waltham, MA, USA) according to the manufacturer's instructions, using 100 ng of fibroblasts total DNA as template. Cycling conditions are illustrated in Table 2:

Table 2 - Primers and PCR cycling conditions

Gene name and ID	OMIM	Primers	PCR cycling conditions
<i>CPT1A</i> – 2328	255120	Exon 12 Forward: 5'TTGGGAGTACGTCATGTCCA3' Reverse: 5'- ATCTGCCCAGGAGTGTTTCAG-3' Exon 13 Forward: 5'TCATGTTGGAGGTTAATGTGTTT3' Reverse: 5'CTGTAAGACTTCAAATGTGTTTCC3'	95°C for 5min followed by 35 cycles (95°C for 30 sec, 59°C for 35 sec, 72°C for 2 min) and 72°C for 10min
<i>DNAJC19</i> -30528	610198	Exon 6 Forward: GCTAAATCTCCCTCAGATAAG-3' Reverse:5'- AAGTTTAGACGGTAGGTAGTATAA-3'	95°C for 5min followed by 30 cycles (95°C for 30 sec, 53°C for 30 sec, 72°C for 2 min) and 72°C for 10min

<i>HADHA</i> - 3030	600890	Exon 15 Forward: 5'- GGTTTCCTCACCCGCATTCTC-3' Reverse: 5'- TCCTTTTACCTCCAGGCTTG-3'	95°C for 5min followed by 30 cycles (95°C for 30 sec, 58°C for 30 sec, 72°C for 2 min) and 72°C for 10min
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PCR products were analysed by 1% agarose gel electrophoresis. The PCR products were subsequently sequenced with the same primers using ABI BigDye™ terminator sequencing kit (ABI, Foster City, CA).

4.6 SDS-PAGE and immunoblotting (I - II)

Western blotting was done as described previously to detect proteins (Prip-Buus et al. 2001)

Antibodies used are listed in Table 3.

Table 3 - Antibodies used for western blotting

Antigen	Antibodies	Reference or source	Publication	Dilutions
CPT1A	Polyclonal Sheep	A kind gift from Carina Prip-Buus, Institut Cochin, Meudon, France).	I	1:2000 dilution in TBST with 5% milk
TFPαβ	Polyclonal Rabbit	A generous gift from Bruce Middleton, University of Nottingham	I	1:2000 dilution in TBST with 5% milk

		Medical School, Nottingham, United Kingdom		
DNAJC19	Polyclonal Rabbit	Proteintech Group, Inc 12096-1-AP	II	1:2000 dilution in TBST with 5% milk

4.7 Homology modelling of Human CPT1A (I)

Homology modelling of the CPT1A structure was done in the SWISS MODEL server using an alignment mode. The template used was the resolved structure of the homolog carnitine acetyltransferase from *Mus musculus*.

4.8 Minisequencing (I)

The 150 unrelated control samples were genotyped using the single nucleotide extension minisequencing method as described previously to detect the carrier frequency of the *CPT1A* gene missense variant in exon 12 in the Finnish population (Syvanen AC et al. 1992). PCR cycling conditions are listed in Table 4

Table 4 - Primers and PCR cycling conditions for Minisequencing

Mutation Exon 12	Primers	PCR cycling conditions
c.1364A>C	Forward: 5'- TGAAGCAGAGAGGTGCAATG -3' Reverse: 5'-biotinylated GAAAATCCAAACCTGGCCAG-3' Miniseq primer: 5'- TTCACGTAGGTGGTTTGACA-3'	95°C for 5min followed by 30 cycles (95°C for 30 sec, 57.1°C for 35 sec, 72°C for 2 min) and 72°C for 10min

4.9 BN-PAGE (II)

We used 2.5µg of enriched mitochondrial protein from skeletal muscle and heart for the BN-PAGE assay. Electrophoresis and semi-dry transfer were done under native conditions as previously described (Antonicka et al. 2006; Lyly et al. 2008). The antibodies were mouse monoclonal antibodies against complexes I (MS111), II (MS204), III (MS302) and IV (MS407) from Mitosciences diluted at 1:10000 or 1:1000 in TBST with 5% milk.

4.10 Publication III methods

The methods used in study III are listed in Table 5 followed by detailed descriptions. The antibodies used for immunocytochemistry are listed in Table 6.

Table 5 - Methods used in Publication III

Methods
iPS cell clones production
RPE differentiation
Immunocytochemistry
Bright field microscopy
Confocal microscopy
Electron microscopy
Fluorescent microscopy
PCR
RT-PCR
<i>In vitro</i> phagocytosis assay
Lipidomics analysis Neutral and Phospholipid staining

4.10.1 hiPSC lines maintenance and reprogramming

Fibroblasts were obtained from skin biopsies of controls (healthy volunteers) and patient then cultured in fibroblast growth medium, Dulbecco's modified eagle medium DMEM (Gibco, Carlsbad, CA, USA), with 10% foetal bovine serum (FBS) (Gibco), 1% GlutMAX (Lonza, Basel, Switzerland), and 1% penicillin and streptomycin (Lonza). Human pluripotent stem cells were cultured on mitomycin-inactivated mouse embryonic fibroblasts (MEFs) in hiPSC medium: Knockout (KO) DMEM (Gibco), supplemented with 20% KO-serum replacement (Gibco), 1% GlutMAX (Gibco) and 6ng/ml basic fibroblasts growth factor (bFGF; Sigma-Aldrich, Missouri, USA). Human pluripotent stem cell lines were passaged enzymatically using 1mg/ml Collagenase IV (Invitrogen, Carlsbad, CA, USA) and passaged onto new mitomycin-treated MEF plates. 293-GPG packaging cells (Ory et al. 1996) were transfected at the confluence of 80-90% with 5 individual pMXs-cDNA vectors, including "Yamanaka" Factors (pMXs_Oct4, pMXs_Sox2, pMXs_Klf4, pMXs_cMyc) and pMXs_dsRed using FugeneHD (Roche, Basel, Switzerland), according to the manufacture instructions. After 24 h transfection the medium was changed to the fibroblast growth medium. On days 4, 5 and 6 the viral supernatants were collected, combined and filtered prior to use through 0.45µm syringe filters (Millipore, Billerica, MA, USA). Fibroblasts were plated between passages 4 to 10 in 6-well plates at a density of 1×10^5 cells/well. Cells were transduced on day 0 and 1 with freshly collected virus supernatants containing equal volumes (2mls each virus) of the 4 retroviruses. On day 3, transduced fibroblasts were washed with phosphate buffered saline (PBS) and the media was changed to fibroblast growth medium. Six days after the transduction, the cells were dissociated by TrypLE Select (Invitrogen) and replated into cell culture plates coated with mitomycin-treated MEFs. Next day, the medium was replaced by the hiPSC medium, which was changed every other day. Colonies were picked 24-30 days after the transduction and transferred to 24 well plates coated with mitomycin-treated MEFs. This cell stage was counted as a passage 1. hiPSC cells were confirmed using teratoma assay.

4.10.2 Differentiation of hiPSC into RPE cells

All pluripotent cell lines were adapted to human foreskin feeder cell layers for at least 8 passages prior to the differentiation. The RPE differentiation was performed as previously described (Vaajasaari et al. 2011).

4.10.3 Immunocytochemistry

Cell fixation and antibody hybridization were performed as described earlier (Vaajasaari et al. 2011). The primary antibodies used and their dilutions are listed in Table 6. A dilution of 1:1500 of donkey anti-mouse IgG and goat anti-rabbit IgG, chicken anti-goat IgG (all Alexa Fluor 488), goat anti-mouse IgG, goat anti-rabbit IgG, donkey anti-sheep IgG (all Alexa Fluor 568) (Molecular probes, Life Technologies, Paisley, UK) were used as secondary antibodies. Images were taken with a Zeiss Axioplan 2 microscope.

4.10.4 RT-PCR

Total RNA was extracted as described earlier from control and patient hiPSC-RPE by using NucleoSpin XS-kit (Macherey Nagel, GmbH & Co, Duren, Germany) (Vaajasaari et al. 2011). Isolated RNA was eluted in 10µl H₂O. The RNA concentration and quality were assessed with a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware). The RNA was DNase I (Invitrogen, Carlsbad, CA, USA) treated to remove any traces of DNA according to the manufacturer's instructions. 5 µg of RNA was used for cDNA synthesis by using the 200U Superscript II RT™ (Life Technologies CA) according to the manufacturer's instructions. cDNA was used as a template in a following PCR reaction, which was performed using Phusion® DNA polymerase (Thermo Fisher, Waltham, MA, USA). Gene specific primer sequence details were based on Vaajasaari (Vaajasaari et al. 2011).

PCR reactions were performed as follows: 98°C, 5 min; 35 x 95°C, 30 sec; 60°C, 30 sec (annealing temperature varied with the primer pair used) (Vaajasaari et al. 2011) and extension at 72°C for 2 min followed by final extension at 72°C for 10 min. PCR products were analyzed on 2% agarose gels with a 100 bp DNA ladder. The bands were visualized with 4.5.2 Basic Program (Bio-Rad Laboratories, Inc., Hercules, CA).

4.10.5 *In vitro* phagocytosis assay

The photoreceptor outer segments (POS) were isolated from the eyes of freshly slaughtered porcines using a continuous sucrose gradient (Vaajasaari et al. 2011). POS were labelled with FITC (0.04 ug/ul; Sigma-Aldrich, Missouri, USA) in 0.1M NaHCO₃ (pH 9.0) were incubated for 1h at RT. Then labelled POS were washed, resuspended in RPE culture medium, and seeded onto collagen IV-coated 0.3cm² BD biocoat culture plate inserts. Cells were incubated at +37°C in 5% CO₂ for 16 h. External fluorescence was removed by trypan-blue treatment for 10 min. Cells were then washed with PBS and

used for immunocytochemistry as described earlier (Vaajasaari et al. 2011). Filamentous actin was stained with 1:40 dilution (Invitrogen, Carlsbad, CA, USA) by incubating for 10 min at RT following several washes with PBS. The nuclei were stained with DAPI that was in mounting media (Vector Laboratories Inc., Burlingame, CA). The images were captured using a confocal microscope (Leica TCS SP2 confocal microscope, Wetzlar, Germany).

4.10.6 Electron microscopy

The RPE cell medium was removed from the collagen coated inserts and the cell layer was cut into pieces, rinsed with PBS, and fixed in 2.5% glutaraldehyde in 0.1M HEPES for 2 h at RT and then transferred to 4% paraformaldehyde for 3 days at 4°C. Further cells were postfixed with 1% osmium tetroxide, dehydrated and embedded in Epon. Images were captured with a Jeol 1200-EXII and Jeol 1400 (Tokyo, Japan) electron microscope.

4.10.7 Lipidomic analysis

First, 170µl 0.9% NaCl-solution was added to the cell pellets from both control and patient hiPSC-RPE, and the samples were then sonicated for 3 min, at 7°C, 40 kHz (Finnsonic m03). For the UPLC-QTOFMS analyses, a standard mixture 1 (10 µl) containing PC (Phosphatidylcholine) (17:0/0:0), PC (17:0/17:0), PE (Phosphatidylethanolamine) (17:0/17:0) and Cer (Ceramide)(d18:1/17:0), (Avanti Polar Lipids, Inc.) and TG (17:0/17:0/17:0) (Larodan Fine Chemicals) was added to 15 µL of cell homogenates. HPLC-grade chloroform and methanol (2:1; 100 µL) was added to the samples, which were then vortexed for 2 min and allowed to stand for 30 min. Subsequently, samples were centrifuged and the lower phase (60µL) was collected and 20µL of internal standard mixture 2 was added. The internal standard mixture 2 contained the labelled lipids PC (16:1/0:0-D₃), PC (16:1/16:1-D₆) and TG (16:0/16:0/16:0-¹³C₃). Detailed information about instrumental conditions were described in Publication III.

4.10.8 Lipid stainings

Lipid (neutral lipids and phospholipids) stainings were performed using HCS LipidTOXTM phospholipidosis and steatosis detection kit according to the manufacturer's instructions. Cells were treated with LipidTOXTM Red phospholipids stain and incubated for 24h. Cells were fixed with 4% paraformaldehyde mixed with

Hoechst staining for 30 min at RT. After washing with PBS, the cells were stained with LipidTOX™ Green neutral lipid (Molecular Probes, Invitrogen Carlsbad, CA, USA) for 30min at RT to detect neutral lipids. Pictures were taken using a Zeiss Axioplan 2 microscope.

4.10.9 Statistical analyses

Statistical analysis were determined by the Student's *t*-test. Data were expressed as mean \pm standard error. Values of *P* < 0.05 were considered significant.

Table 6 - Antibodies used for Immunocytochemistry

Antigen	Antibodies	Reference or source	Dilutions
CPT1A	Polyclonal Sheep	Kind gift from Carina Prip-Buus (Institut Cochin, Meudon, France).	1:300
TFP $\alpha\beta$	Polyclonal Rabbit	Generous gift from Bruce Middleton (University of Nottingham Medical School, Nottingham, UK).	1:400
CRALBP	Mouse monoclonal	Abcam (ab15051)	1:1000
MITF	Rabbit polyclonal	Abcam (ab20663)	
ZO-1	Mouse monoclonal	Invitrogen (ZO1-1A12)	1:250
RPE65	Mouse monoclonal	Millipore (MAB5428)	1:250
ACADM	Rabbit polyclonal	Kind gift from Prof. Jerry Vockley Children's Hospital of Pittsburgh, Pittsburgh, PA, USA	1:400

ACAD9	Rabbit polyclonal	Children's Hospital of Pittsburgh, Pittsburgh, PA, USA	1:400
ACADVL	Mouse monoclonal	Abnova,(M01) Taiwan	1:300
Actin	Goat	Santa Cruz Biotechnology Inc.USA (I -19)	1:300
Na ⁺ /K ⁺ ATPase	Mouse	Abcam (ab76509)	1:50

5 RESULTS AND DISCUSSION

5.1 Characterization of a novel homozygous and compound heterozygous *CPT1A* variants in Finland (I)

Patients with CPT1A deficiency do not show symptoms until there is an increase in energy demand such as during fasting or illness. Bennett (Bennett et al. 2010) and colleagues stated that, the important laboratory findings observed were hypoketotic hypoglycaemia, elevated serum concentrations of liver transaminases, ammonium and total carnitine during increases in physiological stress due to increased energy demand. CPT1A deficiency does not have muscle weakness or cardiomyopathy like other inherited metabolic disorders in FA β O (Ijlst et al. 1998).

We identified six patients in Finland with CPT1A deficiency by gene mutation analyses during an 11 year period. Five were live-born patients. Four out of six patients were from unrelated Finnish families and two were siblings (brothers). All of the patients had the same homozygous variant in exon 12 of the *CPT1A* gene. The initial symptom observed in all patients, was common illness. The main hallmark of the disease is a metabolic hypoketotic hypoglycaemia, hepatopathy, and loss of consciousness or coma; all patients also had hyperlipidaemia and mild hyperammonaemia. However, patient number 6 was an adult who had experienced serious decompensation in childhood due to viral illness. The patient was diagnosed earlier as having HELLP syndrome during her pregnancy (Ylitalo et al. 2005). Patients 1 and 5 were diagnosed after their first attack and have been on low dietary fat therapy treatment since then. Patient 2 (older brother) and patient 3 (younger) were siblings. For all patients (aged from 4 to 34 years), conditions of motor and neuropsychological development have been normal except for patient 1 who had learning disabilities and specific language impairment. Neurological symptoms such as mental retardation, developmental delay and decreased attention span have been reported earlier in CPT1A deficiency (Prasad et al. 2001a).

The sequence analysis of *CPT1A* confirmed a homozygous missense variant c.1364A>C in exon 12 that was found in five patients and compound heterozygosity, c.1364A>C (exon 12)/c.1493A>C (exon 13) in the one remaining patient (patient 5) (I, Figure 1). The locations of the variants are reported in the cDNA sequence (NM_001876.3). Both variants change conserved amino acids (p.Lys455Thr and p.Tyr498Ser) in the N-domain of the protein (I, Figure 3). Gobin and Ijlst groups have earlier reported disease-causing variants in the same region of the *CPT1A* gene (Gobin et al. 2002; Ijlst et al. 1998). Western blotting analyses of the fibroblasts that were obtained from patient 1 with the homozygous variant c.1364A>C (p.Lys455Thr) and compound heterozygous variants in patient 5 showed no protein expression (I, Figure 2). This result was supported by the CPT1A enzyme activity measurements. In addition, the palmitate loading test showed decreased enzyme activity. This has also been supported by our structural model of

CPT1A (based on a template, mouse carnitine acetyltransferase), which predicts that the p.Lys455Thr variant resides in the core of the N-domain that possibly leads to protein degradation due to improper folding. The other variant, p.Tyr498Ser is also predicted to have an impact on protein stability as Tyr498 plays a role in interacting with the N-terminal end of the protein downstream of Ser166 (I, Figure 3).

We used minisequencing analyses to study the carrier frequency of the variant c.1364A>C (exon 12). No carriers were found in 150 healthy unrelated control samples but the allele frequency of *CPT1A* variant in the global population is 0.0002142 (ExAC Browser) whereas in the Finnish population (6614 allele number) the frequency is 0.001966 (ExAC Browser). This is in accordance with the low prevalence of CPT1A deficiency in Finland. However, the patient carrying the compound heterozygote mutation c.1493A>C (p.Tyr498Ser) had a more severe clinical course, which could be due to other factors than the mutation type.

CPT1A is a liver enzyme which also expressed in kidney, leukocytes, fibroblasts. It is a key regulator of fatty acid metabolism (Greenberg et al. 2009). The first variant of CPT1A was reported in the year 1981 (Bougnères et al. 1981) and since then 30 cases of *CPT1A* variants have been reported from across the world (Bennett et al. 2004). However all of the relevant variants detected in the present study had reduced CPT1A expression and enzyme activity in the fibroblasts. Nevertheless, the clinical course among these patients varied from mild hypoglycaemia aggravated by illness to hyperammonaemic coma.

Most studies indicate that CPT1A deficiencies are caused by homozygous variants, but carriers of functional variants could have a higher risk of a disorder in lipid metabolism. Earlier studies have reported *CPT1A* variants in the North American Hutterite community that resulted in hypoketotic hypoglycaemia and signs of encephalopathy. For instance, Prasad and co-workers (Prasad et al 2001b) carried out a pilot study on six patients who belonged to a large extended Hutterite kindred scattered across Canada and the United States. All of them shared a common haplotype on chromosome 11q13 and were homozygous for a common *CPT1A* p.Gly710Glu variant. DNA analyses carried out by Carina Prip-Buus and co-workers (Prip-Buus et al. 2001) on three children (two siblings and their second cousin) belonging to an extended inbred Hutterite kindred revealed that they were homozygous for the c.2129G>A missense variant p.Gly710Glu. The catalytic function of the mutant protein was shown to be impaired (Prip-Buus et al. 2001).

Previous studies indicated that the Canadian and Greenland Inuit, British Colombia First Nations and Alaska Natives come under one population where the *CPT1A* thermolabile gene variant c.1436C>T (p.Pro479Leu) has a high prevalence (Colins et al. 2010, Gessner et al. 2011). Collins and colleagues reported a high allele frequency and rate of homozygosity for the *CPT1A* Pro479Leu variant, which lead to a decreased CPT1A

functional activity, further the patients could be susceptible to decompensation during times of high fever and illness. Another study by Rajakumar and co-workers (Rajakumar et al. 2009) was carried out to determine the population frequency of this variant in a Greenland Inuit community with a population size of 1111. Contrasting results from their study demonstrated that the gene variant c.1436C>T might not necessarily cause CPT1A deficiency and further presentation of similar symptoms as reported by an earlier study might be rather coincidental. Results also indicated that a high frequency of the Leu479 allele that is significantly associated with high plasma levels of HDL-cholesterol and apoA-I, which could possibly act as protecting agents against atherosclerosis. A recent study detected the presence of a *CPT1A* missense variant c.1436C>T (p.Pro479Leu) in arctic populations and further authors suggested that this associated mutation was partly due to various factors including the prevailing cold environment, absence of plant food and in addition a higher intake of fat diet (Clemente et al. 2014).

MS/MS analysis has been an effective technique that has significantly helped in the detection of a number of inborn errors of metabolism (Greenberg et al. 2009). Routine newborn screening of blood spots that includes MS/MS analysis of samples obtained from infants has become a mandatory standard procedure amongst Alaskan and Greenland populations with a high incidence of the c.1436C>T sequence variation in *CPT1A*. Evaluating sensitivities of newborn screening by tandem mass spectrometry were carried out in various studies to identify homozygous infants (Borch et al. 2012, Gessner et al. 2011).

Monique Fontaine and colleagues suggested that a homozygous variant (c.1783 C>T) in *CPT1A* resulted in hepatic CPT deficiency (Fontaine et al. 2012) CPT1A acts as a rate limiting enzyme for β -oxidation in the liver. Thus, reduced CPT1A activity in p.Lys455Thr homozygous fibroblasts would limit FA β O flux and further lead to reduced capacity for hepatic ketogenesis (Greenberg et al. 2009). In contrast low enzyme activity in compound heterozygous (p.Lys455Thr/Tyr498Ser) (Table 7) patients implies that it has blocked the malonyl-CoA mediated regulation and thus leads to a decrease in β -oxidation during lipid metabolism (McGarry et al. 1997; Brown et al. 2001). Interestingly, Luise Borch and colleagues from Denmark have reported a CPT1A patient who had normal levels of plasma free carnitine and acylcarnitines but the patient started showing symptoms and signs at the age of 8 months and was found to have a mild enzyme defect with a novel variant c.167C>T. (Borch et al. 2012).

Table 7 Carnitine Enzyme activity measurement

Patient	Activity (nmol/min.mg)	Control mean \pm SD (n)
Case 5 (compound heterozygous Lys455Thr/Tyr498Ser)	0.00 ↓↓	0.54 \pm 0.20 (12)
Case 6 (homozygous c.1364A>C (Lys455Thr)	0.00 ↓↓	0.54 \pm 0.20 (12)

Our follow up study of five patients (1, 2, 3, 5, and 6) showed improved metabolic compensation with high carbohydrate with the low dietary fat therapy treatment. The dietary therapy consisted of a low fat, high carbohydrates diet and it was provided to all of the patients with the exception of patient 1, who also needed intravenous glucose intake during viral illness. The follow up studies showed good metabolic compensation. However, patient six who was the oldest patient in the group, had delayed dietary therapy that led to various episodes of decompensation with coma. This further suggests that dietary therapy helps in maintaining metabolic compensation; nevertheless strict usage of dietary therapy however remains uncertain. Ingesting high carbohydrate meals and avoidance of fasting possibly aids in the survival of the patient. Furthermore, identifying the disease causing variants and also by keeping a check on the variants existing in other populations can help in the prenatal diagnosis and early therapy.

5.2 DNAJC19 deficiency as a novel cause for early onset dilated cardiomyopathy (II)

This study is the first case report from Europe that describes inherited dilated cardiomyopathic syndrome (DCMA), which is caused by DNAJC19 deficiency outside the previously reported Canadian Dariuslet Hutterite population. DCMA is a novel autosomal recessive condition that occurs in children due to a single gene variant in *DNAJC19* (Murphy et al. 2005; Bowles et al. 2004). DNAJC19 is presumed to play a key role in importing mitochondrial proteins. Here we report a case of two Finnish brothers with a novel homozygous truncating variant c.300delA (NM_145261.3) in the *DNAJC19* gene. Parents of the brothers were heterozygous for the variant, which left them unaffected. The variant is a single nucleotide deletion (A) in exon 6 (II, Figure 1), which does not immediately cause an aminoacid change but produces stop codon 11 aminoacids later thus caused a frameshift and premature truncation of the protein (106

AA). Western blot analysis showed no expression of DNAJC19 protein in the patient fibroblasts (II, Figure 2). Further quantification of ATP and mitochondrial complexes CI, CII and CIV enzymes revealed a reduced enzyme activity in the patient's skeletal muscles, which was indicative of, a mild respiratory chain abnormality. However, the BN-PAGE analysis of their heart and skeletal muscle lysates revealed normal expression of enzyme levels (II, Figure 5). The variant caused a severe onset dilated cardiomyopathy and high excretion of 3-methylglutaconic aciduria type V in urine. The mutation also caused a severe muscle weakness and neurological disturbances. Suddenly at the age of 13 months the younger brother died. The left ventricle of the heart of the deceased patient was found to be noncompacted and dilated. Neuropathological studies revealed mild brain stem atrophy resulting in ataxia.

An earlier study by Davey et al. (2006) reported, DCMA syndrome in the Canadian Dariuslet Hutterite population (Davey et al. 2006). The DNJAC19 patients in the cohort constitute 18 patients: all of whom had onset of DCM before 3 years of age. In addition more than 70% of the affected patients died from either progressive cardiac failure or sudden cardiac death associated with the splice site variant c. IVS3-1G>C in the *DNAJC19* gene (Davey et al. 2006). All the patients over the age of two years had a cerebellar syndrome with ataxia resulting in motor delays. In some male patients, testicular dysgenesis along with impaired androgen and anti-Mullerian hormone synthesis was reported. Other features presented were optic atrophy, elevation in hepatic enzyme levels and mild to borderline non-progressive mental retardation. In the Canadian population, the rate of cardiomyopathy is larger when compared to mitochondrial disorders (Sparkes et al. 2007). Further Sparkes and colleagues have reported 17 patients with dilated cardiomyopathy, which was diagnosed as DCMA syndrome. They were diagnosed at the onset age of 12 months and a range from 1 to 36 months. It was caused by homozygous variant in the *DNAJC19* gene. (Sparkes et al. 2007).

Symptoms of DCMA syndrome include early onset cardiomyopathy, methylglutaconic aciduria and failure of growth which are similar to those observed in Barth syndrome (type II 3-methylglutaconic aciduria) a syndrome is caused by mutations in the X-linked *TAZ* gene on Xq28. The *TAZ* gene encodes tafazzin, a protein that is involved in cardiolipin metabolism in the mitochondria (Davey et al. 2006). Barth syndrome manifests in early infancy with cardiomyopathy, raised 3-MGC and 3-MGA levels, skeletal myopathy, hypotonia, growth delay and neutropenia (Barth et al. 2004, Spencer et al. 2006). Some features, which are not seen in Barth syndrome, are cerebellar symptoms and autosomal recessive inheritance. Both tafazzin and DNAJC19 are mitochondrial proteins. Studies by He and co-workers suggested that the knockdown of tafazzin causes cardiolipin deficiency, which further lead to a reduction in ATP production decreased cardiac contractility, hypertrophy and cell death (He Q et al. 2013). However, only few inherited genetic defects affecting DNAJ proteins and mitochondrial chaperones have been described. Disruption of tafazzin leads to defective OXPHOS and it is also involved in apoptosis and mitochondrial membrane dynamics (Wortmann et al. 2013). In addition, recent studies suggest that Prohibitin (PHB)

/DNAJC19 membrane domains regulate cardiolipin remodelling by tafazzin (Richter-Dennerlein et al. 2014). PHB forms large protein and lipid scaffolds in the inner membrane of mitochondria. PHB's forms large hetero-oligomeric ring complexes composed of PHB1 and PHB2 subunits. PHB complexes are essential for mitochondrial integrity such as mitochondrial morphogenesis and normal life span (Richter-Dennerlein et al. 2014).

Previous studies have also shown that DNAJC19 shares a strong homology with the yeast protein Tim14. Tim 14 localizes in the inner mitochondrial membrane and is involved in the transport of mitochondrial-targeted proteins into the matrix (Mokranjac et al. 2003). This indicates that loss of DNAJC19 in DCMA leads to defects in the import of mitochondrial proteins (Davey et al. 2006), although clear experimental evidence is lacking. Studies indicate that approximately 1000 mitochondrial proteins are encoded by nuclear genes that are synthesized in the cytosol and imported into the mitochondria (Fosslein 2003). Therefore, any defect or mutations may render DNAJC19 into an inactive state or even completely abolished. DNAJC19 may influence the mislocalization of the mitochondrial respiratory chain or matrix proteins. Although it's a reasonable clarification for mitochondrial cytopathy, the precise mechanism still remains unclear (Sparkes et al. 2007) and suggests that mutation could affect many aspects of mitochondrial function. Furthermore studies by Sinha and co-workers (Sinha et al. 2010) demonstrated that human MAGMAS an ortholog of Pam16 in yeast, functions in a similar manner by facilitating the translocation of proteins across the inner mitochondrial membrane. Experiments revealed that MAGMAS could form a stable subcomplex with DNAJC19 through its C-terminal, mutations within DNAJC19 could result in decreased stability of the MAGMAS: DNAJC19 complex, thus causing impairment in both protein importation and cellular respiration.

Another recent study showed that the mitochondrial PHB complexes interact with DNAJC19 (Richter-Dennerlein et al. 2014). Cells lacking in either DNAJC19 or PHB2, showed clear indications of alterations in the acyl chain composition of cardiolipin reminiscent of cells lacking TAZ suggests that DNAJC19 could be involved in the translocation of TAZ into the mitochondrial inner matrix. Alterations in the acyl chain composition of cardiolipin could interfere with membrane rearrangements, which would lead to disturbances in the morphology of cristae in DNAJC19-deficient mitochondria. Biochemical analyses results from this study were quite discordant with the results of an earlier study, which DNAJC19 associates with TIM23 translocases (Davey et al. 2006). Therefore, the absence of either DNAJC19 or PHB2 results in conditions that include impaired cell growth, a disturbed mitochondrial ultrastructure, and similar transcriptional responses, all of which suggests that the PHB/DNAJC19 complex represents the functional active structure (Richter-Dennerlein et al. 2014).

5.3 Evaluation of hiPSC derived RPE cells obtained from a LCHADD patient (III)

One of the outcomes of deficiencies in FA β O defects is LCHAD deficiency. The manifestation of the disease is retinopathy, neuropathy, and cardiomyopathy. Among all FA β O defects retinopathy occurs only in LCHAD deficient patients (Tyni et al. 1998a). If this condition is untreatable then it leads to a loss of vision. Underlying pathogenic mechanisms for the cause of pigmentary retinopathy in LCHADD patients is still unknown. Earlier Tyni et al., used isolated porcine RPE cells and immortalized RPE (ARPE 19) cells to study the pathogenesis but no standard physiological model has been proposed so far (Tyni et al. 1998a). We introduced an *in vitro* hiPSC model in this study, which helps to investigate the pathogenic mechanism that causes pigmentary retinopathy. The concept pertaining to this paper was designed based on our earlier histopathological and ophthalmological findings (Tyni et al. 1998a, Tyni et al. 1998b, Tyni et al. 2012) obtained from LCHADD patient eye.

Current treatments of LCHAD deficiency do not cure retinopathy progression, the pigment clumping at the level of RPE and the central pigmentation may disappear and result in the progressive loss of central RPE and choroid, and leaving the central sclera bare which could potentially lead to blindness. We generated hiPSC from a LCHADD patient's fibroblasts and from controls to understand the pathogenesis of retinopathy. They showed similar characteristic features to the hESCs (III Figure 3). Moreover, the control and LCHADD patient derived hiPSC-RPE cells produced a monolayer of hexagonal polyhedral shaped cells with distinct nuclei with a mosaic like pattern. They both expressed the RPE cell markers particularly, ZO1, CRALBP, MITF, Na⁺/K⁺ ATPase as confirmed by immunocytochemistry and RT-PCR (III Figure 4). In general, the patient cells were smaller in size when compared to the control cells. Further there was a significant difference observed between the samples upon measuring the cell volume. Interestingly, the patient cells also had low pigmentation in all three hiPSC cell lines produced.

For RPE, the phagocytosis of photoreceptor outer segments (POS) is an important function and the basis for proper vision. Incubation with porcine POS control and patient hiPSC-RPE demonstrated the ability of the RPE cells to phagocytose, thereby demonstrating their functionality *in vitro* (III Figure 5). No specific difference was observed between the cells. With ZO-1 staining, the patient hiPSC-RPE cells appeared circular and possibly formed incomplete cell-to-cell adhesion, which indicates that the intercellular gaps might weaken the RPE layer. Instead, the control hiPSC-RPE cells had a hexagonal shape, and formed regular tight junctions. The RPE monolayer constitutes the outer blood retina barrier, thus the integrity of adherens junctions is vital for normal functioning of the neural retina (Tyni et al. 2004). In addition, previous studies suggested that both the choriocapillaris and RPE cells are affected early in its course (Tyni et al 1998b). Consequently, disruption of adherens junctions may degenerate the RPE layer in patients, which is supported in clinical histopathological findings (Tyni et al. 1998b).

Pigmentation in RPE cells is due to the melanin component in organelles called melanosomes. When subjected to electron microscopy (III, Figure 7), the amount of melanosomes appeared to be fewer in patient cells, in comparison to control cells. Numerous lipid accumulations were also observed in patient cells when compared to control cells. In addition, Oil-red-O staining showed vast dispersed lipids in patient cells whereas in the controls it appeared like small clumps in a particular region (unpublished data) (Figure 8). Further, the patient cells had huge accumulations of neutral staining lipids and a weak expression of phospholipids upon LipidTOXTM staining (III, Figure 8A). LipidTOXTM staining usually detects the intracellular accumulation of neutral lipids and phospholipids in the cells. Neutral staining lipids expression in control cells was weak and phospholipids had a very faint expression. Lipidomics data generated by mass spectrometry found a significant increase of triglycerides (TG) in patient cells compared to control cells (III, Figure 8B). The neutral staining results were concordant with lipidomics analyses, which suggested a two-fold increase in triglyceride accumulation. Intracellular lipid accumulation could possibly occur when there was a mismatch between free fatty acid import and utilization. It has also been shown that TG accumulation in non-adipose cells could be a response to an acute palmitate overload, which could be a cellular defence against lipotoxicity (Listenberger et al. 2003). Similar findings have been detected earlier in stress-induced inflammatory response in human corneal epithelial cells exposed to increasing osmolarity (Robciuc et al. 2012). Lipidomics analyses showed significant up-regulation of TGs in the stress-induced cells, particularly polyunsaturated molecular species. Relatively, large TGs were highly up-regulated, which is in agreement with our current results. Non-adipose cells have a limited capacity for lipid storage, therefore excess free FAs may have impaired normal cell signalling and thus caused cellular dysfunction and apoptotic cell death (Schaffer et al. 2003).

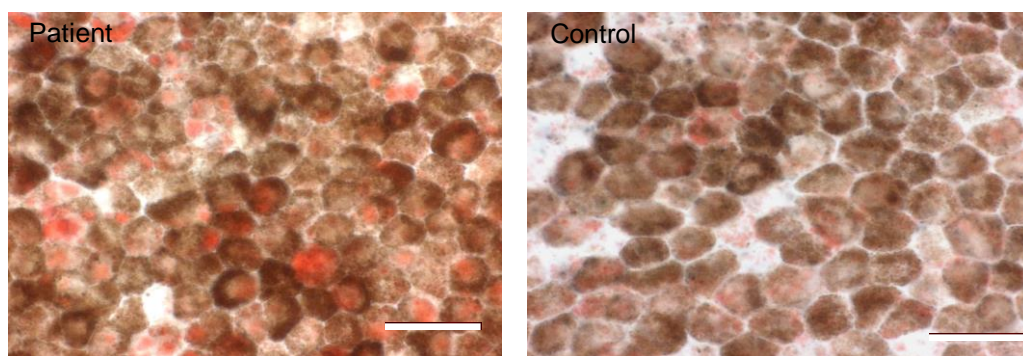


Figure 8 – Oil red O staining in Patient and Control hiPSC –RPE cells Magnification 63x Scale bar = 10 μ m

Retinopathy manifestation in defective mitochondrial FA β O is surprising as in the brain glucose is considered to be the main source of energy in the retina (Berman 1991). Clinical and histopathological findings in retinopathy of LCHAD deficiency indicate

that the choriocapillaris and RPE cells are affected early in its course, usually by the age of two years (Tyni et al. 1998b). The possible mechanism of retinal damage suggested in literature was the accumulation of toxic metabolites of impaired long chain fatty acid oxidation (Gillingham et al 2005). Further Gillingham and co-workers suggested that the severe progression of chorioretinopathy and decreased retinal function on the fundus images associated with increased levels of long chain 3-hydroxyacylcarnitines. Interestingly, DHA deficiency was detected in plasma of the one year old LCHADD patient. DHA is considered to be abundant fatty acid in retinal photoreceptor membrane phospholipid and also suggested that DHA deficiency is associated with retinal dysfunction in rhesus monkeys and human infants (Harding et al. 1999). Further, Harding hypothesis (Harding et al. 1999) suggests that the DHA deficiency also contributes to retinopathy in LCHADD patients. Low levels of DHA have also been observed in some LCHAD deficiency children. (Gillingham et al. 2005).

Tyni and co-workers used human retinal sections and cultured porcine RPE cells to explain the energy metabolism of the RPE cells in relation to the retinopathy condition in LCHAD deficiency (Tyni et al. 2004). Their results convincingly explains the role of mitochondrial FA β O in RPE such as the expression of MTP and other enzymes such as ACAD9 in RPE and other layers of retina such as ganglion cells, inner segments of photoreceptors in RPE. The expression and localization of β -oxidation enzymes such as ACADVL, ACADM, ACAD9, CPT1A and MTP $\alpha\beta$ expression in hiPSC-RPE cells in our study were positive (III, Figure 6). Now, it is quite clear that the current results are concordant with our earlier findings (Tyni et al. 2002, 2004; Roomets et al. 2006).

Our findings from the patients strongly suggest that retinopathy of LCHAD deficiency was due to the degeneration of the RPE layer from the accumulation of toxins resulting from the lack of HADHA. In addition, earlier studies (Tyni et al. 2012) from a 5 year old patient eye images showed grades of stage 2 pigment deposits and RPE atrophy and there were two different stages of retinopathy observed. Thus the hypopigmentation and pigment clumping in the macula in stage 2 and total atrophy of the posterior pole, posterior staphyloma in stage 4 appears to be due to disrupted RPE. These stages support the current findings such as reduced cell size, less melanosomes (hypopigmentation), accumulated toxins and disrupted cell-cell contacts (ZO1) crucial for RPE function.

There are many studies associated with using iPSC disease modelling techniques. For instance, studies reported on patient-derived iPSCs of retinal diseases such as retinitis pigmentosa (RP) (Jin et al. 2011) and gyrate atrophy (GA) (Meyer et al. 2011). RP is an inherited human eye disease that is caused by the degeneration of photoreceptors (Jin et al. 2011). In contrast GA is a rare autosomal recessive disease caused by a mutation in the gene ornithine δ aminotransferase (*OAT*), that primarily affects the RPE and thereby causes blindness. Recently, GA patient specific RPE derived from iPSCs were developed that showed a disease specific functional defect i.e., very low OAT activity in RPE cells (Meyer et al. 2011) thereby demonstrating the use of patient specific iPSCs in understanding disease pathogenesis and also to test new therapeutic drugs. Other studies

include Best disease, which is an inherited degenerative disease of the human macula that causes loss of central vision (Singh et al. 2013).

The hiPSC-RPE cells are the most promising candidates to be used as they can be produced easily with established protocols using various substrates. They were found to show mature phenotypes that exhibit key physiological functions *in vitro*. (Singh et al. 2013). Schwartz and colleagues used hESC-RPE cell transplantation observed improved vision in age-related macular degeneration and stargardt's macular dystrophy patients with long-term safety. This indicates the success of using hESC-derived cells in clinical trials. (Schwartz et al. 2015). Recently researchers in Japan also announced the approval for transplanting autologous iPSC-RPE into clinic for patients after performing safety tests. They changed the law to permit these studies; they are classed as a clinical iPS cell pilot study for 6 patients. This has been halted due to the presence of oncogene activation in the second patient iPS cells.

In summary, we were able to produce the first *in vitro* LCHADD disease model for pigmentary retinopathy. This cellular model illustrated novel early pathogenic changes in LCHADD retinopathy- a gross disruption of the RPE cell morphology, less pigmentation, few melanosomes, defective tight junctions and an excessive accumulation of TGs early upon differentiation and – all were fully consistent with patient findings. These changes are likely to cause fatal consequences for the main functions of the RPE.

7 CONCLUSIONS AND FUTURE PROSPECTS

The research presented in this PhD thesis identified novel disease-causing variants in *CPT1A* and *DNAJC19* in the Finnish population. This series of studies worked towards the implementation of hiPSC in order to understand the disease pathogenesis of LCHADD. Furthermore, these studies might help in providing knowledge for future therapies for the above mentioned FA β O disorders.

Our results give insight into the clinical manifestation of *CPT1A* protein deficiency, which were based on biochemical analyses, molecular characterization and structural analysis. The carrier frequency of the *CPT1A* variant in the Finnish population showed no single carrier in the healthy controls, but the allele frequency of *CPT1A* variant in the global populations is 0.0002142 (ExAC Browser), whereas in Finnish population (6614 allele number) the frequency is higher at 0.001966 (ExAC Browser). The structural study of the *CPT1A* protein can help clinicians in identifying or predicting the consequences of new variants. Advanced techniques such as next generation sequencing that include exome and whole genome sequencing may be able to provide in depth study of multiple variants of *CPT1A*. The implementation of screening of neonates and infants in paediatric medicine is an important aspect to consider as it can aid in early detection of disease-causing variants and can facilitate early treatment. Newborn screening for defects in long-chain FA β O defects has already reduced mortality and also helps in identifying variants in mildly affected patients. Additionally, treating *CPT1A* patients with dietary therapy is an important step to consider, as it would improve metabolic levels and a better clinical status.

The DCMA cases due to *DNAJC19* deficiency are the first patients diagnosed in Europe. Effective screening of the urinary organic acid analysis together with the analysis of cardiac disease is needed well before undergoing molecular genetic analysis. Further studies are required to identify the phenotype-genotype relationship and elucidate a deeper understanding of the underlying pathogenic mechanisms that cause DCMA syndrome. Performing population based screening tests in the near future will further help to understand the cardiac phenotype and other mitochondrial disorders.

Current developments in regenerative medicine are providing valuable techniques to study the pathogenesis of various diseases. The iPSC technology provides a revolutionary platform in regenerative medicine that facilitates, in understanding various diseases and their mechanisms. This particular approach has helped to circumvent the ethical issues that relate to use of hESC. However, other challenges remain as in the case of hESC such as tumour formation.

In our study, differentiated RPE cells from LCHADD patient fibroblasts appear to show a disrupted RPE layer, which is a key component of vision. Currently, there are no

specific drugs to treat LCHADD. Optimal low fat diet therapy is the only treatment for LCHAD deficiency in patients that helps in slowing the progression of chorioretinopathy and vision loss. In addition, supplementation of DHA may improve visual acuity in children with LCHADD. Execution of hiPSC techniques on LCHADD patient specific fibroblasts, revealed a new pathway in understanding the pathogenesis of the disease mechanism study. The disease specific hiPSC derived cells acts as a bridge between the clinical phenotype and molecular or cellular mechanisms along with other strategies including drug screening or developing novel therapeutic agents. Many labs have used this method to generate iPSC from patient fibroblasts cells. These findings aid in the newly emerging field of mitochondrial FA β O disorders and assist in the development of novel treatments for clinical trials.

The further identification and elucidation of molecular mechanisms of the above mentioned cases will help in understanding the full pathogenicity of the disease and should illuminate the path to develop potential treatments and medical care for the patients. Genetic counselling is also an important aspect for early diagnosis and better treatment and these should also benefit from further research.

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